SUPPORT FOR THE AMENDMENTS

A substitute specification has been submitted in order to make grammatical changes. A clean copy and a marked-up copy of the substitute specification is submitted herewith.

Claim 1 has been amended to make a clarifying amendment.

No new matter is believed to have been added to this application by these amendments.

REMARKS

Claims 1-24 are active in this application. Favorable reconsideration is respectfully requested.

The rejection of the claims under 35 U.S.C. §112, first paragraph (written description), is respectfully traversed.

The present application provides a detailed description of a method for screening a microorganism that can metabolize a carbon source in a liquid medium containing L-glutamic acid at a saturation concentration and the carbon source at a specific pH. See page 8, line 27 to page 12, line 7 of the present specification. Therefore, even in the case where a microorganism is one other than *Enterobacter agglomerans*, one skilled in the art can understand that such a microorganism can be obtained by the screening method described in the present application. Accordingly, Applicants had possession of the claimed invention at the time the present application and, therefore, the present application satisfied the written description requirement of 35 U.S.C. §112, first paragraph, at the time of filing. Withdrawal of this ground of rejection is respectfully requested.

The rejection of the claims under 35 U.S.C. §112, first paragraph (enablement), is respectfully traversed.

The present application provides a detailed description of a method for screening a microorganism that can metabolize a carbon source in a liquid medium containing L-glutamic acid at a saturation concentration and the carbon source at a specific pH. See page 8, line 27 to page 12, line 7 of the present specification. Therefore, even in the case where a microorganism is one other than *Enterobacter agglomerans*, one skilled in the art can understand that such a microorganism can be obtained by the screening method described in the present application.

In addition, Applicants submit the following publications D1-D4, which demonstrate that microorganisms other than *Enterobacter agglomerans* alos have similar pathways and enzymes related to L-glutamic acid biosynthesis.

D1 (U.S. 5,846,790) shows that the gdh gene, gltA gene, and the ppc gene from *Brevibacterium lactofermentum* (coryneform bacterium), where the genes code for the enzymes related to L-glutamic acid biosynthesis.

D2 (EP 0670370B1) shows that the enzymes of L-glutamic acid biosynthesis from *E. coli*.

D3 (*Escherichia coli* and *Salmonella typhimurium*) p. 156-157 and p. 302-318 (1987)) shows L-glutamic acid biosynthesis pathways of *E. coli* and *S. typhimurium*.

D4 (J. Bacteriol., 181, 6679-6688 (1999)) shows the carbon metabolism of *E. coli*.

In view of D1-D4, a microorganism other than a microorganism belonging to the genus *Enterobacter* is also considered to have similar pathways and enzymes for glutamic acid biosynthesis as compared to microorganisms belonging to the genus *Enterobacter*. Therefore, one skilled in the art can isolate can isolate such enzymes from a microorganism other than a microorganism belonging to the genus *Enterobacter* by conventional methods such as PCR.

In view of the detailed teachings in the present specification and the state of the art as demonstrated by publications D1-D4, one can obtain and use the claimed microorganism without undue experimentation. Accordingly, the claims are enabled. Withdrawal of this ground of rejection is respectfully requested.

The rejection of the claims under 35 U.S.C. §112, second paragraph, is believed to be obviated by the amendment submitted above. Claim 1 has been amended to specify that the L-glutamic acid is accumulated in the medium. Withdrawal of this ground of rejection is respectfully requested.

The obviousness-type double patenting rejection over Applicant's co-pending application serial No. 10/077,751 is respectfully traversed.

In the co-pending application, the claims have been amended as suggested by the Examiner. In amended Claim 1 of the co-pending application, the medium contains an organic acid containing 1, 2, or 3 carbon atoms, the amount of the organic acid in the medium is 0.4 g/L or less, and the amount of organic acid in the medium does not inhibit the growth of the microorganism. On the other hand, such limitations are not recited in the present invention, and are not described or suggested by the present invention. Accordingly, Claims 10-13 and 15-24 of the present application are not obvious over the claims of the co-pending application. Withdrawal of this ground of rejection is respectfully requested.

In response to the Examiner's request for information under 35 U.S.C. §132 and 37 C.F.R. §1.78(c), Applicants confirm that the co-pending application serial No. 10/077,751 was commonly owned at the time of the invention described in the present application was made.

A substitute specification has been submitted. If any additional changes to the specification are believed to be necessary, the Examiner is invited to contact the undersigned.

Applicants submit that the present application is in condition for allowance. Early notice to this effect is earnestly solicited.

Respectfully submitted,

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D1

United States Patent 5,846,790
Kimura , et al. December 8, 1998

Methods of producing L-lysine and L-glutamic acid by fermentation

Abstract

A mutant strain having an ability to produce L-glutamic acid in the absence of any biotin action-suppressing agent in a medium containing an excessive amount of biotin is obtained by giving temperature sensitivity with respect to a biotin action-suppressing agent to a coryneform L-glutamic acid-producing bacterium. This strain is cultivated in a liquid medium to produce and accumulate L-glutamic acid in the medium. A mutant strain having an ability to produce L-lysine and L-glutamic acid in the absence of any biotin action-suppressing agent in a medium containing an excessive amount of biotin is obtained by giving temperature sensitivity with respect to a biotin action-suppressing agent and giving L-lysine productivity to a coryneform L-glutamic acid-producing bacterium. This strain is cultivated in a liquid medium to simultaneously produce and accumulate L-lysine and L-glutamic acid in the medium.

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Field of Search: 435/252.1,110,111,115,840,843,252.32

References Cited [Referenc d By]

U.S. Patent Documents

3971701	Jul., 1976	Takinami et al.	435/111.
4334020	Jun., 1982	Nakazawa et al.	435/110.

Primary Examiner: Lilling; Herbert J.
Attorney, Agent or Firm: Oblon, Spivak, McClelland, Maier & Neustadt, P.C.

Claims

What is claimed is:

1. A method of producing L-glutamic acid by fermentation without biotin-action suppressive agents, comprising the steps of:

cultivating a mutant strain in a liquid medium;

raising the temperature at an intermediate stage of the cultivation to a temperature where said mutant strain is sensitive to a biotin action-suppressing agent at a concentration at which growth of said mutant strain at 31.5.degree. C. is approximately equivalent to that in the absence of said biotin action-suppressing agent;

producing and accumulating L-glutamic acid in the medium; and

collecting L-glutamic acid from the medium,

said mutant strain being a coryneform L-glutamic acid-producing bacterium, having a temperature-sensitive mutation with respect to a biotin action-suppressing agent produced by applying a mutation treatment to a coryneform L-glutamic acid-producing bacterium, conducting a replication method in a growth medium containing a biotin-action-suppressing agent and selecting a strain which is temperature sensitive at 37.degree. C. in said growth medium but not temperature sensitive in media without said biotin-action-suppressing agent, and having the ability to produce L-glutamic acid in the absence of any biotin action-suppressing agent in a medium containing an excessive amount of biotin.

2. A method of producing L-glutamic acid according to claim 1, wherein the biotin action-suppressing agent is polyoxyethylene sorbitan monopalmitate.

3. A method of producing L-glutamic acid according to claim 1, wherein protein expression levels in said mutant strain of one or more genes selected from the group consisting of glutamate dehydrogenase gene, citrate synthase gene, phosphoenolpyruvate dehydrogenase gene and isocitrate dehydrogenase gene have been enhanced.

4. A method of producing L-lysine and L-glutamic acid by fermentation without biotin-action suppressing agents, comprising the steps of:

cultivating a mutant strain in a liquid medium;

raising the temperature at an intermediate stage of the cultivation to a temperature where said mutant strain is sensitive to a biotin action-suppressing agent at a concentration at which growth of said mutant strain at 31.5.degree. C. is approximately equivalent to that in the absenc of said biotin action-suppressing agent;

producing and accumulating L-lysine and L-glutamic acid in the medium; and

collecting them from the medium, said mutant strain being a coryneform L-glutamic acid-producing bacterium, having a mutation to give L-lysine productivity and a temperature-sensitiv mutation with respect to a biotin action-suppressing agent produced by applying a mutation treatment to a coryneform L-glutamic acid-producing bsacterium, conducting a replication method in a growth medium containing a biotin-action-suppressing agent and selecting a strain which is temperature sensitive at 37.degree. C. in said growth medium but not temperature sensitive in media without said biotin-action-suppressing agent, and having the ability to produce L-lysine and L-glutamic acid in the absence of any biotin action-suppressing agent in a medium containing an excessive amount of biotin.

5. A method of producing L-lysine and L-glutamic acid according to claim 4, wherein the biotin action-suppressing agent is polyoxyethylene sorbitan monopalmitate.

6. A mutant strain of coryneform bacteria, having a temperature-sensitive mutation with respect to a biotin action-suppressing agent produced by applying a mutation treatment to a coryneform L-glutamic acid-producing bacterium conducting a replication method in a growth medium containing a biotin-action-suppressing agent and selecting a strain which is temperature sensitive at about 37.degree. C. in said growth medium but not temperature sensitive in media without said biotin-action-suppressing agent, and having the ability to produce L-glutamic acid in the absence of any biotin action-suppressing agent in a medium containing an excessive amount of biotin.

7. A mutant strain according to claim 6, wherein the biotin action-suppressing agent is polyoxyethylene sorbitan monopalmitate.

8. A mutant strain according to claim 6 having a mutation to give L-lysine productivity and having the ability to produce both L-lysine and L-glutamic acid in the absence of any biotin action-suppressing agent in a medium containing an excessive amount of biotin.

9. A mutant strain according to claim 8, wherein the biotin action-suppressing agent is polyoxyethylene sorbitan monopalmitate.

10. A method of breeding mutant coryneform strains having the ability to produce L-glutamic acid in the absence of any biotin action-suppressing agent in a medium containing an excessive amount of biotin, comprising:

giving temperature sensitivity with respect to a biotin action suppressing agent to a coryneform L-glutamic acid-producing bacterium produced by applying a mutation treatment to a coryneform L-glutamic acid-producing bsacterium, conducting a replication method in a growth medium containing a biotin-action-suppressing agent and selecting a strain which is temperatur sensitive at 37.degree. C. in said growth medium but not temperature sensitive in media without said biotin-action-suppressing agent.

11. A method of breeding mutant coryneform strains having the ability to produce both L-lysine and L-glutamic acid in the absence of any biotin action-suppressing agent in a medium c ntaining an excessive amount of biotin, comprising:

giving temperature sensitivity with respect to a biotin action-suppressing agent produced by applying a mutation treatment to a coryneform L-glutamic acid-producing bsacterium, conducting a replication method in a growth medium containing a biotin-action-suppressing agent and selecting a strain which is temperature sensitive at about 37.degree. C. in said growth medium but not temperature sensitive in media without said biotin-action-suppressing agent and giving L-lysine productivity to a coryneform L-glutamic acid-producing bacterium.

12. A method of producing L-glutamic acid according to claim 1, wherein the temperature is raised to about 33.degree.-40.degree. C. at said intermediate stage of the cultivation.

13. A method of producing L-glutamic acid according to claim 1, wherein the temperature is raised to about 37.degree.-40.degree. C. at said intermediate stage of the cultivation.

14. A method of producing L-lysine and L-glutamic acid according to claim 4, wherein the temperature is raised to about 33.degree.-40.degree. C. at said intermediate stage of the cultivation.

15. A method of producing L-lysine and L-glutamic acid according to claim 4, wherein the temperature is raised to about 37.degree. 40.degree. C. at said intermediate stage of the cultivation.

Description

TECHNICAL FIELD

The present invention relates to methods of producing L-lysine and L-glutamic acid by fermentation. L- lysine is widely used as a feed additive, etc., and L-glutamic acid is widely used as a material for seasonings, etc.

BACKGROUND ART

L-lysine and L-glutamic acid have been hitherto industrially produced by fermentatative methods by using coryneform bacteria belonging to the genus Brevibacterium or Corynebacterium having abilities to produce these amino acids. In these methods, it is known that the coryneform bacteria require biotin for their growth, while L-glutamic acid is not accumulated if an excessive amount of biotin exists in a medium. Therefore, any one of the following methods has been adopted in the conventional method of producing L-glutamic acid. Namely, cultivation is conducted in a medium in which the concentration of biotin is restricted, or cultivation is conducted such that a surfactant or a lactam antibiotic as a biotin action-suppressing agent is allowed to be contained in a medium at an initial or intermediate stage of cultivation in the case of use of the medium containing a sufficient amount of biotin.

However, especially when a material such as waste molasses, which is inexpensive but contains an excessive amount of biotin, is used as a carbon source in a medium, the biotin action-suppressing agent, which is required to be added to the medium, has been a cause to increase the production cost.

On the other hand, the following methods are known for simultaneous production of L-lysine and L-glutamic acid by fermentation. Namely, an L-lysine-producing bacterium is cultivated under a condition for L-glutamic acid production, or an L-lysine-producing bacterium and an L-glutamic acid-producing bacterium are mixed and cultivated (Japan se Patent Laid-open No. 5-3793).

However, in the method in which an L-lysine-producing bacterium is cultivated under a condition for L-glutamic acid production to simultaneously produce L-lysine and L-glutamic acid by fermentation, the condition for L-glutamic acid production is either that a biotin-auxotrophic bacterium belonging to the genus Brevibacterium or Corynebacterium is cultivated in a medium containing a low concentration of biotin, or that it is cultivated such that a surfactant or a lactam antibiotic is allowed to be contained in a medium at an initial or intermediate stage of cultivation in the case of the medium containing a sufficient amount of biotin. Especially when a material such as waste molasses, which is inexpensive but contains an excessive amount of biotin, is used as a carbon source in a medium, the surfactant or the lactam antibiotic as a biotin action-suppressing agent to be added to the medium has been a cause to increase the production cost.

Further, in the method in which an L-lysine-producing bacterium and an L-glutamic acid-producing bacterium are mixed and cultivated, there has been a problem that control of cultivation is difficult, and fermentation results are unstable.

DISCLOSURE OF THE INVENTION

An object of the present invention is to provide a method of producing L-glutamic acid inexpensively and stably by fermentation in which no biotin action-suppressing agent is added even when a material such as waste molasses containing an excessive amount of biotin is used as a carbon source in a medium.

Another object of the present invention is to provide a method of simultaneously producing L-lysine and L-glutamic acid inexpensively and stably by fermentation in which no biotin action-suppressing agent is added even when a material such as waste molasses containing an excessive amount of biotin is used as a carbon source in a medium.

As a result of vigorous investigations in order to achieve the aforementioned objects, the present inventors have found that a mutant strain obtained by giving temperature sensitivity to a biotin action-suppressing agent to a conventionally used coryneform L-glutamic acid-producing bacterium produces and accumulates a considerable amount of L-glutamic acid even in a medium containing an excessive amount of biotin without adding any surfactant or antibiotic. Further, the present inventors have found that a mutant strain derived by giving temperature-sensitivity to a biotin action-suppressing agent to an L-lysine-producing bacterium originating from a coryneform L-glutamic acid-producing bacterium produces and accumulates considerable amounts of both L-lysine and L-glutamic acid even in a medium containing an excessive amount of biotin without containing any surfactant or antibiotic. Thus the present invention has been completed.

Namely, the present invention lies in a method of producing L-glutamic acid by fermentation comprising the steps of cultivating a mutant strain in a liquid medium, producing and accumulating L-glutamic acid in the medium, and collecting it from the medium, the mutant strain originating from a coryneform L-glutamic acid-producing bacterium, having temperature sensitive mutation to a biotin action-suppressing agent, and having an ability to produce L-glutamic acid in the absence of any biotin action-suppressing agent in any medium containing an excessive amount of biotin.

In another aspect, the present invention lies in a method of producing L-lysine and L-glutamic acid by fermentation comprising the steps of cultivating a mutant strain in a liquid medium, producing and accumulating L-lysine and L-glutamic acid in the medium, and collecting them from the medium, the mutant strain originating from a coryneform L-glutamic acid-producing bacterium, having mutation to give L-lysine productivity and temperature sensitive mutation to a

bi tin action-suppressing agent, and having an ability t produce L-lysine and L-glutamic acid in the absenc of any biotin action-suppressing agent in any medium containing an excessive amount of biotin.

According to another aspect of the present invention, there is provided a mutant strain originating from a coryneform L-glutamic acid-producing bacterium, having temperature sensitive mutation to a biotin action-suppressing agent, and having an ability to produce L-glutamic acid in the absence of any biotin action-suppressing agent in a medium containing an excessive amount of biotin. This mutant strain will be sometimes referred to below as "first mutant strain of the present invention".

According to another aspect of the present invention, there is provided a mutant strain originating from a coryneform L-glutamic acid-producing bacterium, having mutation to give L-lysine productivity and temperature sensitive mutation to a biotin action-suppressing agent, and having an ability to produce both L-lysine and L-glutamic acid in the absence of any biotin action-suppressing agent in a medium containing an excessive amount of biotin. This mutant strain will be sometimes referred to below as "second mutant strain of the present invention".

According to another aspect of the present invention, there is provided a method of breeding mutant strains having an ability to produce L-glutamic acid in the absence of any biotin action-suppressing agent in a medium containing an excessive amount of biotin comprising giving temperature sensitivity to a biotin action-suppressing agent to a coryneform L-glutamic acid-producing bacterium.

According to another aspect of the present invention, there is provided a method of breeding mutant strains having an ability to produce both L-lysine and L-glutamic acid in the absence of any biotin action-suppressing agent in a medium containing an excessive amount of biotin comprising giving temperature sensitivity to a biotin action-suppressing agent and L-lysine productivity to a coryneform L-glutamic acid-producing bacterium.

The present invention will be explained in detail below.

<1> PREPARATION OF MUTANT STRAIN TEMPERATURE-SENSITIVE TO BIOTIN ACTION-SUPPRESSING AGENT ORIGINATING FROM L-GLUTAMIC ACID-PRODUCING BACTERIUM, AND PRODUCTION OF L-GLUTAMIC ACID

•! Preparation of Mutant Strain Temperature-Sensitive to Biotin Action-Suppressing Agent Originating from L-Glutamic Acid-Producing Bacterium

Conventional and known L-glutamic acid-producing bacteria originating from coryneform L-glutamic acid-producing bacteria, when they are cultivated in a medium containing an excessiv amount of biotin of not less than 10 .mu.g/L, substantially produce no L-glutamic acid in a culture liquid, unless a biotin action-suppressing agent such as surfactants and antibiotics is allowed to be contained in the medium at an initial or intermediate stage of cultivation. The first mutant strain of the present invention has an ability to produce L-glutamic acid even when it is cultivated in a liquid medium containing an excessive amount of biotin, without allowing any biotin action-suppressing agent to be contained in the medium. Namely, the first mutant strain of th present invention is a mutant strain originating from a coryneform L-glutamic acid-producing bacterium, having temperature-sensitive mutation to a biotin action-suppressing agent, and having an ability to produce L-glutamic acid in the absence of any biotin action-suppressing agent in a medium containing an excessive amount of biotin.

The mutant strain as described above can be induced by giving temperature sensitivity to a biotin action-suppressing agent to a glutamic acid-producing bacterium originating from a

coryneform L-glutamic acid-producing bacterium. The biotin action-suppressing agent includes, for example, surfactants and antibiotics.

The surfactants include, for example, saturated fatty acid such as lauric acid, myristic acid, stearic acid, and palmitic acid; fatty acid ester type nonionic surfactants such as glycerol fatty acid ester, sorbitan fatty acid ester, sucrose fatty acid ester, polyethylene glycol fatty acid ester, polyethylene glycol polypropylene glycol fatty acid ester, and polyoxyethylene sorbitan fatty acid ester; and N-acyl amino acids such as N-palmitoylglycine, N-palmitoylalanine, N-palmitoylvaline, N-palmitoylleucine, N-palmitoylthreonine, N-palmitoylmethionine, N-palmitoylaspartic acid, N-palmitoylglutamic acid, N-myristoylglutamic acid, N-stearoylglutamic acid, N,N'-dipalmitoylornithine, and N,N'-dipalmitoyllysine.

The antibiotics include, for example, lactam antibiotics such as penicillin and cephalolysine.

In general, the growth of coryneform L-glutamic acid-producing bacteria is inhibited by the presence of a biotin action-suppressing agent at a certain concentration or more. In the present invention, the temperature sensitivity to a biotin action-suppressing agent means a property such that growth is remarkably inhibited, as compared with that in the absence of the biotin action-suppressing agent, at a cultivation temperature of 33.degree.-37.degree. C., preferably not less than 34.degree. C. in the presence of the biotin action-suppressing agent at a maximum concentration at which approximately equivalent growth to that in the absence of the biotin action-suppressing agent is observed at 31.5.degree. C. (optimum growth temperature). Specifically, a property as defined as follows is intended. Namely, when the influence exerted by the biotin action-suppressing agent is investigated at temperatures of 31.5.degree. C. and 33.degree.-370.degree. C., a degree of relative growth in the presence of the biotin action-suppressing agent at each concentration is calculated provided that each growth in the absence of the biotin action-suppressing agent at each temperature is regarded as 100, and a maximum concentration at which the degree of relative growth at 31.5.degree. C. is not less than 80 is determined, then the degree of relative growth at a temperature of 33.degree.-37.degree. C. is not more than 50 in the presence of the biotin action-suppressing agent at the maximum concentration.

The coryneform L-glutamic acid-producing bacteria referred to in the present invention includ bacteria having been hitherto classified into the genus Brevibacterium but united as bacteria belonging to the genus Corynebacterium at present (Int. J. Syst. Bacteriol., 41, 255 (1981)), and include bacteria belonging to the genus Brevibacterium closely relative to the genus Corynebacterium. Therefore, the mutant strain used in the present invention can be induced from the following coryneform L-glutamic acid-producing bacteria belonging to the genus Brevibacterium or Corynebacterium. In this specification, when the L-glutamic acid productivity is not referred to, the bacteria belonging to the genera Corynebacterium and Brevibacterium are simply referred to as "coryneform bacteria".

Corynebacterium acetoacidophilum	ATCC 13870
Corynebacterium acetoglutamicum	ATCC 15806
Corynebacterium callunae	ATCC 15991
Corynebacterium glutamicum	ATCC 13032
(Brevibacterium divaricatum)	ATCC 14020
(Brevibacterium lactofermentum)	ATCC 13869
(Corynebacterium lilium)	

ATCC 15990
(Brevibacterium flavum)
ATCC 14067
Corynebacterium melassecola
ATCC 17965
Brevibacterium saccharolyticum
ATCC 14066
Brevibacterium immariophilum
ATCC 14068
Brevibacterium roseum ATCC 13825
Brevibacterium thiogenitalis
ATCC 19240
Microbacterium ammoniaphilum
ATCC 15354
Corynebacterium thermoaminogenes
AJ12340 (FERM
BP-1 539)

The bacterial strain having temperature sensitivity to the biotin action-suppressing agent can be obtained by applying a mutation treatment such as ultraviolet light irradiation, X-ray irradiation, radiation irradiation, and mutating agent treatments to a bacterial strain as described above, followed by conducting a replica method on an agar plate medium containing the biotin action-suppressing agent. Namely, the growth state of a parent strain in the presence of several concentrations of the biotin action-suppressing agent is observed at a cultivation temperature of 33.degree.-37.degree. C., preferably not less than 34.degree. C. to determine a maximum concentration of the biotin action-suppressing agent at which growth is recognized. A mutant strain may be separated which cannot grow or has a remarkably lowered growth speed in the presence of the biotin action-suppressing agent at the maximum concentration at the same temperature as that used above.

The temperature sensitivity to the biotin action-suppressing agent is given to the coryneform L-glutamic acid-producing bacterium as described above. Thus the mutant strain can be bred which has the ability to produce L-glutamic acid in the absence of the biotin action-suppressing agent in a medium containing an excessive amount of biotin.

·! Preparation of Mutant Strain Temperature-Sensitive to Biotin Action-Suppressing Agent Originating from L-Glutamic Acid-Producing Bacterium by Means of Genetic Recombination

Alternative methods for obtaining mutant strains temperature-sensitive to the biotin action-suppressing agent may be available other than the method based on the mutation treatment described above. For example, a gene relevant to resistance to the biotin action-suppressing agent is obtained from a coryneform L-glutamic acid-producing bacterium, and the gene is subjected to a mutation treatment in vitro to obtain a mutant type gene which gives the resistance to the biotin action-suppressing agent in a temperature sensitive way. Next, a corresponding wild type gene on chromosome is substituted with the mutant type gene by using an already established technique for homologous recombination. Thus a mutant strain temperature-sensitive to the biotin action-suppressing agent can be obtained.

A gene relevant to surfactant resistance will be described below as a gene relevant to the resistance to the biotin action-suppressing agent. A gene relevant to surfactant resistance originating from a coryneform bacterium may be isolated by:

(1) obtaining a surfactant-sensitive mutant strain belonging to the coryneform bacteria having increased sensitivity to a surfactant;

(2) ligating various chromosomal DNA fragments of a wild type coryneform bacterium with a vector capable of operation in the coryneform bacteria to prepare various recombinant DNA's;

(3) introducing the various recombinant DNA's into the surfactant-sensitive mutant strain belonging to the coryneform bacteria to perform transformation;

(4) selecting a strain with lost surfactant sensitivity, that is, a strain with increased surfactant resistance from transformed strains;

(5) recovering recombinant DNA from the transformed strain with lost surfactant sensitivity; and

(6) analyzing the structure of a chromosomal DNA fragment of the wild type coryneform bacterium ligated with the vector.

The chromosomal DNA fragment of the wild type coryneform bacterium thus obtained contains the gene relevant to the surfactant resistance originating from the coryneform bacterium. This gene at least relates to a mechanism of the coryneform bacterium to produce L-glutamic acid in a medium containing a surfactant. At the same time, it also has a possibility of common relation to production of L-glutamic acid by means of addition of penicillin or restriction of biotin.

In the item (1) described above, the "surfactant-sensitive mutant strain belonging to the coryneform bacteria having increased sensitivity to surfactant" refers to a mutant strain belonging to the coryneform bacteria with deteriorated growth in a medium in which a surfactant exists at a concentration at which no influence is exerted on growth of the wild type coryneform bacterium. For example, when polyoxyethylene sorbitan monopalmitate is used as the surfactant, the surfactant-sensitive mutant strain belonging to the coryneform bacteria makes poor growth as compared with the wild strain if the surfactant is added to a medium at a concentration of 0.1-1 mg/dl. On the other hand, the wild type coryneform bacterium makes no observable change in growth even in a medium in which the surfactant is added at a concentration of 0.1-1 mg/dl. The concentration of the surfactant required for L-glutamic acid production decreases, as compared with an ordinary case, when such a surfactant-sensitive mutant strain is cultivated to produce L-glutamic acid by adding the surfactant. It is postulated that cells of the surfactant-sensitive mutant strain have a state which is approximate to a state of cells of the wild strain exposed to the surfactant.

Preparation of Gene Relevant to Surfactant Sensitivity

In order to obtain the surfactant-sensitive mutant strain belonging to the coryneform bacteria, a method described in Japanese Patent Publication No. 52-24593 can be used. Namely, a mutation-inducing treatment such as ultraviolet light irradiation, X-ray irradiation, radiation irradiation, and mutating agent treatments is applied to a coryneform glutamic acid-producing bacterium to obtain a strain which cannot grow on an agar medium containing a surfactant in an amount with which the parent strain grows.

The surfactant-sensitive mutant strain belonging to the coryneform bacteria is specifically exemplified by Brevibacterium lactofermentum AJ11060, which is disclosed in Japanese Patent Publication No.59-10797.

The method for preparing various chromosomal DNA fragments of the wild type coryneform bacterium is as follows. Namely, the wild type coryneform bacterium is cultivated in a liquid medium, and chromosomal DNA is recovered from collected cells in accordance with a method of Saito et al. (H. Saito and K. Miura, Biochem. Biophys. Acta, 72, 619 (1963)). Recovered chromosomal DNA is digested with a restriction enzyme. A variety of DNA fragments can be

prepared by conducting a reacti n under a condition to incompletely decompose DNA by using an enzyme of a four–nucleotide recognition typ as the restriction enzyme.

The vector capable of operation in the coryneform bacteria is, for example, a plasmid capable of autonomous replication in the coryneform bacteria. Specifically, it can be exemplified by the followings.

(1) pAM330 (see Japanese Patent Laid–open No. 58–67699)

(2) pHM1519 (see Japanese Patent Laid–open No. 58–77895)

(3) pAJ655 (see Japanese Patent Laid–open No. 58–192900)

(4) pAJ611 (see the same)

(5) pAJ1844 (see the same)

(6) pCG1 (see Japanese Patent Laid–open No. 57–134500)

(7) pCG2 (see Japanese Patent Laid–open No. 58–35197)

(8) pCG4 (see Japanese Patent Laid–open No. 57–183799)

(9) pCG11 (see the same)

In order to ligate the vector capable of operation in the coryneform bacteria with various chromosomal DNA fragments of the wild type coryneform bacterium to prepare various recombinant DNA's, the vector is digested with the same restriction enzyme as the restriction enzyme used to digest chromosomal DNA, or with a restriction enzyme which generates a terminal sequence complementary to a terminal sequence of various chromosomal DNA fragments. The digested vector is usually ligated with the chromosomal DNA fragments by using a ligase such as T4 DNA ligase.

In order to introduce the various recombinant DNA's into the surfactant–sensitive mutant strain belonging to the coryneform bacteria, a procedure may be carried out in accordance with conventional and reported transformation methods. For example, it is possible to use a method in which recipient cells are treated with calcium chloride to increase permeability of DNA as reported for Escherichia coli K–12 (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159 (1970)), and a method in which competent cells are prepared from cells at a proliferating stage to introduce DNA as reported for Bacillus subtilis (Duncan, C. H., Wilson, G. A. and Yound, F. E., Gene, 1, 153 (1977)). Alternatively, it is also possible to apply a method in which DNA recipient cells are converted into a state of protoplasts or spheroplasts which easily incorporate recombinant DNA to introduce recombinant DNA into DNA recipients as known for Bacillus subtilis, actinomycetes, and yeast (Chang, S and Choen, S. N., Molec. Gen. Genet., 168, 111 (1979); Bibb, M. J., Ward, J. M. and Hopwood, O. A., Nature, 274, 398 (1978); Hinnen, A., Hicks, J. B. and Fink, G. R., Proc. Natl. Acad. Sci. USA, 75, 1929 (1978)).

As for the protoplast method, a sufficiently high frequency can be obtained even by using the method used for Bacillus subtilis described above. However, as disclosed in Japanese Patent Laid–open No. 57–183799, it is also possible to utilize a method in which DNA is allowed to be incorporated in a state in which protoplasts of coryneform bacterial cells contact with divalent metal ion and one of polyethylene glycol or polyvinyl alcohol. Incorporation of DNA can be also facilitated by addition of carboxymethyl cellulose, dextran, Ficoll, Pluronic F68 (Serva) and the like, in place of polyethylene glycol or p lyvinyl alcohol. An electric pulse method (see Japanese

Patent Laid-open No. 2-207791) was used in Example of the present invention as a method for transformation.

A method for selecting strains with lost surfactant sensitivity from transformed strains will be described below.

DNA fragments having sizes of about 4-6 Kbp obtained by partially digesting chromosomal DNA of a wild strain of a coryneform bacterium with a restriction enzyme Sau3AI are ligated with a plasmid vector capable of autonomous proliferation in both Escherichia coli and coryneform bacteria to produce recombinant DNA's which are introduced into competent cells of Escherichia coli DH5 strain (produced by Takara Shuzo Co., Ltd.) or the like. Transformed strains are cultivated to construct a gene library of the wild strain of the coryneform bacterium.

The surfactant-sensitive mutant strain AJ11060 is transformed with recombinant DNA's contained in the gene library described above. Obtained transformants are once spread on M-CM2G agar plates (containing glucose 5 g, polypeptone 10 g, yeast extract 10 g, NaCl 5 g, DL-methionine 0.2 g, agar 15 g, and chloramphenicol 4 mg in 1 l of pure water, pH 7.2) containing no surfactant to form about 40,000 colonies. The colonies are replicated on M-CM2G plates containing 30 mg/L of a surfactant (Tween 40) to obtain those exhibiting good growth on the M-CM2G plates containing the surfactant. Thus strains with lost surfactant sensitivity can be obtained.

The same method as the method for preparing chromosomal DNA of wild type coryneform bacteria may be used to recover recombinant DNA from a transformed strain with lost surfactant sensitivity. Namely, the transformed strain is cultivated in a liquid medium, and recombinant DNA can be recovered from collected cells in accordance with a method of Saito et al (H. Saito and K. Miura, Biochem. Biophys. Acta, 72, 619 (1963)).

The structure of the chromosomal DNA fragment of the wild type coryneform bacterium ligated with the vector is analyzed, for example, as follows. An entire nucleotide sequence of the chromosomal DNA fragment is determined by a dideoxy method which is an ordinary method for nucleotide sequencing. The structure of DNA is analyzed to determine existing positions of enhancer, promoter, operator, SD sequence, leader peptide, attenuator, initiation codon, termination codon, open reading frame and so on.

One of genes relevant to surfactant resistance originating from a coryneform bacterium obtained as described above in Example 3 described below was designated as "dtsR gene". This dtsR gene at least has a sequence from 467-469th ATG to 1985-1987th CTG of a nucleotide sequence shown in SEQ ID NO: 1 in Sequence Listing. An amino acid sequence which can be encoded by this gene is shown in SEQ ID NOS: 1 and 2 in Sequence Listing. Another ATG (nucleotide Nos. 359-361) exists in the same frame at a position upstream from the aforementioned 467-469th ATG. It is impossible to deny the possibility that the additional ATG is the initiation codon. However, it is postulated that the aforementioned 467-469th ATG is the initiation codon according to analysis of a consensus sequence existing in a region upstream from this gene. Namely, it is postulated that the dtsR gene codes for a peptide having an amino acid sequence comprising amino acid Nos. 37-543 in the amino acid sequence shown in SEQ ID NO: 2. This peptide was designated as "DTSR protein". When the amino acid sequence of the DTSR protein and the nucleotide sequence of the dtsR gene are referred to in the specification and claims of the present invention, they may be generally described by using 467-469th ATG as the initiation codon. However, the possibility of 359-361th ATG as the initiation codon should be also taken into consideration. For example, when it is intended to introduce the dtsR gene into a bacterium belonging to the genus Corynebacterium to enhance its expression, it is assumed that a sequence comprising nucleotide Nos. 467-1987 in the nucleotide sequence shown in SEQ ID NO: 1 may be expressed. However, it will be easily understood by those skilled in the art that the

DTSR protein can be correctly expressed regardless of any ATG as the initiation codon, if a coding region and an upstream region of the nucleotid sequence shown in SEQ ID NO: 1 including nucleotide Nos. 359–466 are introduced into the bacterium belonging to the genus Corynebacterium. Wh n the dtsR gene is expressed in bacterial cells, Met at the N–terminal encoded by the initiation codon is occasionally cut by aminopeptidase.

As shown in Example 3 described below, it has been revealed that the amino acid sequence of the DTSR protein has homology to a protein having been already reported. The protein is described as .beta.–subunit of propionyl–CoA carboxylase (PPC) protein in Proc. Natl. Acad. Sci. USA, vol. 83 (1986) 8049–8053; Proc. Natl. Acad. Sci. USA, vol. 83 (1986) 4864–4868; and Gene, vol. 122 (1992) 199–202. Any of these literatures has no description which suggests that the protein relates to glutamic acid productivity.

Propionyl–CoA carboxylase is an enzyme which catalyzes a reaction in a metabolic pathway to convert .alpha.–ketoglutarate into succinyl–CoA through 2–hydroxyglutarate, propionyl–CoA, D–methylmalonyl–CoA, and L–methylmalonyl–CoA. This metabolic pathway is probably a pathway to bypass a reaction catalyzed by .alpha.–ketoglutarate dehydrogenase in the TCA cycle. In addition, propionyl–CoA carboxylase is an enzyme which uses biotin as a coenzyme. According to these facts, it is suggested that the reaction catalyzed by propionyl–CoA carboxylase, and the aforementioned metabolic pathway or a part thereof including the reaction also [illegible] surfactant resistance. Therefore, it is highly probable that the genes relevant to the surfactant resistance include genes coding for .alpha.–subunit of propionyl–CoA carboxylase, or other enzymes or subunit thereof for catalyzing each of the reactions of the aforementioned metabolic pathway, in addition to the dtsR gene. Further, the present inventors have found that a DTSR protein–deficient strain requires oleic acid for cultivation. Acetyl–CoA carboxylase which uses biotin as a coenzyme has a similar structure to that of propionyl–CoA carboxylase. Thus genes coding for enzymes or subunits thereof for catalyzing each of reactions of the fatty acid metabolism pathway probably relate to the surfactant resistance, as well. The "mutation to exhibit temperature sensitivity to the biotin action–suppressing agent" of the present invention may also include mutation on such genes.

Preparation of Mutant Type dtsR Gene–Introduced Strain by Gene Substitution

The temperature–sensitive mutation to surfactants can be also given by generating mutation on the gene relevant to the surfactant resistance as represented by the dtsR gene obtained as described above. Namely, a procedure may be carried out by introducing mutation in vitro into the obtained gene to prepare a mutant type gene which provides a temperature–sensitive function of its gene product, and substituting the wild type gene existing on chromosome with the mutant type gene by means of homologous gene recombination. Gene substitution by homologous recombination has been already established. It is possible to utilize, for example, a method using linear DNA, or a method using temperature–sensitive plasmid.

Specifically, the modification of the dtsR gene into a mutant type gene is performed by causing substitution, deletion, insertion, addition, or inversion of one or more nucleotides in a nucleotide sequence of a coding region or a promoter region of the dtsR gene by means of a site–directed mutagenesis method (Kramer, W. and Frits, H. J., Methods in Enzymology, 154, 350 (1987)), or a treatment with a chemical reagent such as sodium hypochlorite and hydroxylamine (Shortle, D. and Nathans, D., Proc. Natl. Acad. Sci. U.S.A., 75, 270 (1978)).

The site–directed mutagenesis method is a method of using synthetic oligonucleotides, which is a technique to enable introduction of optional substitution, deletion, insertion, addition, or inversion only into optional and limited base pairs. When this method is utilized, at first a plasmid having a cloned dtsR gene with determined nucleotide sequence is denatured to prepare single–strand DNA. Next, a synthetic oligonucleotide compl mentary to a portion intended to cause

mutation is used. However, the synthetic oligonucleotide is not allowed to have a completely complementary sequence, but allowed to have optional base substitution, deletion, ins rtion, addition, or inversion. The single-strand DNA is annealed with the synthetic oligonucleotide having optional base substituti n, deletion, insertion, addition, or inversion. A complete double-strand plasmid is synthesized by using T4 ligase and Klenow fragment of DNA polymerase I, and it is introduced into competent cells of Escherichia coli. Some transformants thus obtained have plasmids containing genes in which optional base substitution, deletion, insertion, addition, or inversion is fixed. Similar techniques to introduce gene mutation include a recombinant PCR method (PCR Technology, Stockton press (1989)).

The method using a chemical reagent treatment is a method in which mutation having base substitution, deletion, insertion, addition, or inversion is randomly introduced into a DNA fragment by treating the DNA fragment containing an objective gene directly with sodium hypochlorite, hydroxylamine or the like.

The method for substituting a normal gene on chromosome of a coryneform L-glutamic acid-producing bacterium with the mutation-introduced gene thus obtained includes a method which utilizes homologous recombination (Experiments in Molecular Genetics, Cold Spring Harbor Laboratory press (1972); Matsuyama, S. and Mizushima, S., J. Bacteriol., 162, 1196 (1985)). In the homologous recombination, when a plasmid or the like which has a sequence having homology t a sequence on chromosome is introduced into a bacterial cell, recombination takes place at a certain frequency at a position of the sequence having homology, and the entire introduced plasmid is incorporated into the chromosome. When further recombination takes place thereafter at a position of the sequence having homology on the chromosome, the plasmid falls off from the chromosome. At this time, the mutation-introduced gene is sometimes preferentially fixed on the chromosome depending on the position at which recombination takes place, and the original normal gene falls off from the chromosome together with the plasmid. By selecting such a bacterial strain, it is possible to obtain a bacterial strain in which the normal gene on the chromosome is substituted with the mutation-introduced gene having base substitution, deletion, insertion, addition, or inversion.

•! Improvement in L-Glutamic Acid Productivity of Surfactant Temperature-Sensitive Mutant Strain by Enhancement of Genes of Glutamic Acid Biosynthesis System

The L-glutamic acid productivity of the surfactant temperature-sensitive mutant strain of the L-glutamic acid-producing bacterium can be improved by enhancing genes of the glutamic acid biosynthesis system. The genes of the glutamic acid biosynthesis system having been enhanced in cells include, for example, phosphofructokinase of the glycolytic pathway (PFK, Japanese Patent Laid-open No. 63-102692), phosphoenolpyruvate carboxylase of an anaplerotic pathway (PEPC, Japanese Patent Laid-open Nos. 60-87788 and 62-55089), citrate synthase of the TCA cycle (CS, Japanese Patent Laid-open Nos. 62-201585 and 63-119688), aconitate hydratase (ACO, Japanese Patent Laid-open No. 62-294086), isocitrate dehydrogenase (ICDH, Japanese Patent Laid-open Nos. 62-166890 and 63-214189), and glutamate dehydrogenase which catalyzes amination reaction (GDH, Japanese Patent Laid-open No. 61-268185).

Several methods as described below may be available in order to obtain the aforementioned genes.

(1) A mutant strain is obtained in which a characteristic character is exhibited as a result of occurrence of mutation on an objective gene, and the character disappears by introducing the objective gene. A gene which complements the character of the mutant strain is obtained from chromosome of a coryneform bacterium.

(2) If an objective gene has been already obtained from another organism, and its nucleotide

sequence has been clarified, then the objective gene is obtained by means of a technique of hybridization using DNA in a region having high homology as a probe.

(3) If a nucleotide sequence of an objective gene has been revealed fairly in detail, then a gene fragment containing the objective g n is obtain d by means of a PCR method (Polymerase Chain Reaction Method) using chromosome of a coryneform bacterium as a template.

The chromosome used herein can be obtained in accordance with the method of Saito et al. described above (H. Saito and K. Miura, Biochem. Biophys. Acta 72, 619 (1963)). Any host-vector system available in the coryneform bacteria may be used, and those having been described above may be used. The method in the aforementioned item (3), which is effective when the nucleotide sequence has been clarified, was used in Example of the present invention described below.

When a gene is obtained in accordance with the methods of the items (2) and (3) described above, an objective gene occasionally has no unique promoter. In such a case, the objective gene can be expressed by inserting a DNA fragment having promoter activity in the coryneform bacteria into a position upstream from the objective gene. In order to enhance expression of an objective gene, it may be available that the objective gene is ligated at a position downstream from a strong promoter. Strong promoters among promoters which operate in cells of the coryneform bacteria include, for example, lac promoter, tac promoter, and trp promoter of Escherichia coli (Y. Morinaga, M. Tsuchiya, K. Miwa and K. Sano, J. Biotech., 5, 305-312 (1987)). In addition, trp promoter of the coryneform bacteria is also a preferable promoter (Japanese Patent Laid-open No. 62-195294). The trn promoter of the coryneform bacteria was used for expression of PEPC gene in Example of the present invention described below.

•! Production of L-Glutamic Acid by Surfactant Temperature-Sensitive Mutant Strain Originating from L-Glutamic Acid-Producing Bacterium

The method of producing L-glutamic acid according to the present invention comprises cultivating, in a liquid medium, the surfactant temperature-sensitive mutant strain originating from the L-glutamic acid-producing bacterium obtained as described above, producing and accumulating L-glutamic acid in the medium, and collecting it from the medium.

An ordinary nutrient medium containing a carbon source, a nitrogen source, inorganic salts, growth factors, etc. is used as the liquid medium for the cultivation of the aforementioned mutant strain according to the present invention. The mutant strain of the present invention has an ability to produce L-glutamic acid without allowing any biotin action-suppressing agent to be contained in a medium even in the case of cultivation in any liquid medium containing an excessive amount of biotin.

Carbohydrates such as glucose, fructose, sucrose, waste molasses, starch hydrolysate; alcohols such as ethanol and glycerol; and organic acids such as acetic acid may be used as the carbon source. Ammonium sulfate, ammonium nitrate, ammonium chloride, ammonium phosphate, ammonium acetate, ammonia, peptone, meat extract, yeast extract, corn steep liquor, etc. may be used as the nitrogen source. When a mutant strain having an auxotrophy is used, a required substance is added as a preparation or a natural material containing it.

Fermentation is performed for 2-7 days under an aerobic condition achieved by shaking cultivation, aerating and agitating cultivation or the like while maintaining pH of culture liquid at 5-9. pH ic adjucted by ucing urea, calcium carbonate, ammonia gas, aqueouc ammonia and the like. The cultivation temperature is 24.degree.-37.degree. C. However, a better result is obtained by initiating cultivation at about 31.5.degree. C., and raising the temperature to 33.degree.-40.degree. C., preferably about 37.degree. C. at an intermediate stage of the cultivation. Nam ly,

the bacterium is sufficiently proliferated in the vicinity of a temp rature optimum for growth, and then the temperature is raised during cultivation. Thus production of L-glutamic acid is initiated without adding any biotin action-suppressing agent, and L-glutamic acid is produced and accumulated in a considerable amount in a culture liquid.

The present inventors have found that a DTSR protein-deficient strain has an ability to produce L-glutamic acid without allowing any biotin action-suppressing agent to be contained in a medium even in the case of cultivation in any liquid medium containing an excessive amount of biotin. The DTSR protein-deficient strain requires oleic acid for its cultivation. However, the surfactant temperature-sensitive strain does not require addition of oleic acid when it is cultivated at an ordinary temperature, namely about 31.5.degree. C.

Collection of L-glutamic acid produced and accumulated in the culture liquid may be carried out in accordance with an ordinary method. For example, a method of ion exchange resin, a method of crystallization, etc. may be used. Specifically, L-glutamic acid is adsorbed and separated by using an anion exchange resin, or crystallized by neutralization.

<2> PREPARATION OF SURFACTANT TEMPERATURE-SENSITIVE MUTANT STRAIN HAVING L-LYSINE PRODUCTIVITY, AND PRODUCTION OF L-LYSINE AND L-GLUTAMIC ACID

When a conventional and known L-lysine-producing bacterium originating from a coryneform L-glutamic acid-producing bacterium is cultivated in a medium containing an excessive amount of biotin of not less than 10 .mu.g/L, it produces and accumulates only L-lysine in a culture liquid and produces substantially no L-glutamic acid as well in the same manner as the L-glutamic acid-producing bacterium described above, unless a biotin action-suppressing agent such as surfactants or antibiotics is allowed to be contained in the medium at an initial or intermediate stage of cultivation. The mutant strain to be used in the present invention has an ability to produce both L-lysine and L-glutamic acid without allowing any biotin action-suppressing agent to be contained in a medium even when it is cultivated in any liquid medium containing an excessive amount of biotin. Such a mutant strain has the L-lysine productivity in addition to the property of the surfactant temperature-sensitive mutant strain of the L-glutamic acid-producing bacterium described above. Namely, the second mutant strain of the present invention is a mutant strain originating from a coryneform L-glutamic acid-producing bacterium, having mutation to give L-lysine productivity and temperature-sensitive mutation to a biotin action-suppressing agent, and having an ability to produce both L-lysine and L-glutamic acid in the absence of any biotin action-suppressing agent in a medium containing an excessive amount of biotin.

The L-lysine productivity can be usually given by using resistant mutation to S-(2-aminoethyl)-L-cysteine (hereinafter sometimes abbreviated as "AEC") (Japanese Patent Publication No. 48-28078). Other L-lysine-producing mutant strains include, for example, a mutant strain which requires amino acid such as L-homoserine for its growth (Japanese Patent Publication No. 56-6499); a mutant strain which exhibits resistance to AEC and requires amino acids such as L-leucine, L-homoserine, L-proline, L-serine, L-arginine, L-alanine, and L-valine (U.S. Pat. Nos. 3,708,395 and 3,825,472); an L-lysine-producing mutant strain which exhibits resistance to DL-.alpha.-amino-.epsilon.-caprolactam, .alpha.-amino-lauryllactam, aspartate-analog, sulfa drug, quinoid, N-lauroylleucine; an L-lysine-producing mutant strain which exhibits resistance to inhibitors of oxyaloacetate decarboxylase or respiratory system enzymes (Japanese Patent Laid-open Nos. 50-53588, 50-31093, 52-102498, 53-9394, 53-86089, 55-9783, 55-9759, 56-32995 and 56-39778, and Japanese Patent Publication Nos. 53-43591 and 53-1833); an L-lysine-producing mutant strain which requires inositol or acetic acid (Japanese Patent Laid-open Nos. 55-9784 and 56-8692); an L-lysine-producing mutant strain which exhibits sensitivity to fluoropyruvic acid or temperature not less than 34.degree. C. (Japanese Patent Laid-open Nos. 55-9783 and 53-86090); and an L-lysine producing mutant strain (U.S. Pat. No. 4,411,997).

The second mutant strain of the present invention can be induced, for xample, by giving temperature sensitivity to a biotin action-suppressing ag nt such as surfactants and antibiotics to an L-lysine-producing bacterium originating from a coryneform L-glutamic acid-producing bacterium.

The temperature sensitivity can be given in the same manner as the introduction of temperature sensitivity into the first mutant strain of the present invention as described in the item <1> described above. Namely, the bacterial strain having temperature sensitivity to the biotin action-suppressing agent can be obtained by applying a mutation treatment such as ultraviolet light irradiation, X-ray irradiation, radiation irradiation, and mutating agent treatments to a coryneform glutamic acid-producing bacterium having L-lysine productivity, followed by conducting a replica method on an agar plate medium containing the biotin action-suppressing agent. Namely, the growth state of a parent strain in the presence of several concentrations of the biotin action-suppressing agent is observed at a cultivation temperature of 33.degree.-37.degree. C., preferably not less than 34.degree. C. to determine a maximum concentration of the biotin action-suppressing agent at which growth is recognized. A mutant strain may be separated which cannot grow or has a remarkably lowered growth speed in the presence of the biotin action-suppressing agent at the maximum concentration at the same temperature as that used above. Alternatively, as shown in the item <1> •!, a mutant strain temperature-sensitive to the biotin action-suppressing agent may be obtained by genetic recombination.

Alternatively, the second mutant strain to be used in the present invention can be also obtained by previously inducing a mutant strain temperature-sensitive to the biotin action-suppressing agent from a coryneform L-glutamic acid-producing bacterium, followed by giving L-lysine productivity to the mutant bacterial strain.

The temperature sensitivity to the biotin action-suppressing agent and the L-lysine productivity are given to the coryneform L-glutamic acid-producing bacterium as described above. Thus the mutant strain can be bred which has the ability to produce both L-lysine and L-glutamic acid in the absence of any biotin action-suppressing agent in a medium containing an excessive amount of biotin.

As described in the item <1> •!, the productivity of L-glutamic acid can be improved for the second mutant strain of the present invention by enhancing genes of the glutamic acid biosynthesis system as described above. In the same manner, the L-lysine productivity can be improved by enhancing genes of the lysine biosynthesis system.

Known examples of the genes of the lysine biosynthesis system having been enhanced in cells include a gene coding for aspartokinase .alpha.-subunit protein or .beta.-subunit protein in which concerted feedback inhibition by L-lysine and L-threonine is substantially desensitized (WO94/25605 International Publication Pamphlet), a wild type phosphoenolpyruvate carboxylase gene originating from a coryneform bacterium (Japanese Patent Laid-open No. 60-87788), a gene coding for wild type dihydrodipicolinate synthetase originating from a coryneform bacterium (Japanese Patent Publication No. 6-55149), etc.

An ordinary nutrient medium containing a carbon source, a nitrogen source, inorganic salts, growth factors, etc., which is similar to that used for cultivation of the first mutant strain described above, is used as a liquid medium for cultivation of the second mutant strain of the present invention. The second mutant strain of the present invention has an ability to produce L-lysine and L-glutamic acid without allowing any biotin action suppressing agent to be contained in a medium even in the case of cultivation in any liquid medium containing an excessive amount of biotin.

Fermentation is performed for 2–7 days under an aerobic condition achieved by shaking cultivation, agitating and aerating cultivation or the like while maintaining pH of culture liquid at 5–9. pH is adjusted by using urea, calcium carbonate, ammonia gas, aqueous ammonia nd the like. The cultivation temperature is 24.degree.–37.degree. C. However, a better result is obtained by initiating cultivation at about 31.5.degree. C., and raising the temperature to 33.degree.–40.degree. C., preferably about 37.degree. C. at an intermediate stage of the cultivation. Namely, L–lysine is mainly produced at about 31.5.degree. C., but the rate of L–glutamic acid production is increased by raising the temperature during the cultivation. By utilizing this phenomenon, it is possible to control the ratio of L–lysine to L–glutamic acid in a culture liquid to be finally obtained as desired.

An ordinary method may be used for collecting L–lysine and L–glutamic acid produced and accumulated in the culture liquid. For example, a method of ion exchange resin, a method of crystallization, etc. may be used. When the method of ion exchange resin is used, L–lysine is firstly adsorbed and separated from the culture liquid by using a cation exchange resin, and then L–glutamic acid is adsorbed and separated by using an anion exchange resin, or crystallized by neutralization. When L–lysine and L–glutamic acid are used as a mixture, it is of course unnecessary to separate these amino acids with each other.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows influences exerted by PESP on growth of Brevibacterium lactofermentum AJ13029 and its parent strain ATCC 13869 at 31.5.degree. C. and 35.degree. C.

FIG. 2 shows surfactant resistance of Brevibacterium lactofermentum AJ11060 having an introduced plasmid containing dtsR gene.

FIG. 3 shows influences exerted by PESP on growth of Brevibacterium lactofermentum AJ12993 at 31.5.degree. C. and 34.degree. C.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention will be more specifically explained below with reference to Examples.

EXAMPLE 1

Preparation of Mutant Strain Temperature–Sensitive to Biotin Action–Suppressing Agent Originating from Coryneform L–Glutamic Acid–Producing Bacterium

1. Measurement of Sensitivity of L–Glutamic Acid–Producing Bacterium to Biotin Action–Suppressing Agent by Replica Method

Sensitivity of Brevibacterium lactofermentum ATCC 13869 to polyoxyethylene sorbitan monopalmitate (PESP) was measured as follows in accordance with a replica method.

Brevibacterium lactofermentum ATCC 13869 was cultivated overnight at 31.5.degree. C. on a CM2B agar plate medium having a composition shown in Table 1 to obtain bacterial cells. They were suspended in sterilized physiological saline, seeded on the aforementioned agar plate medium, and cultivated at 31.5.degree. C. for 20–30 hours to form colonies. They were replicated on a CM2B agar medium added with each concentration of PESP, and cultivated at 35.degree. C. for 20–30 hours to observe the growth state.

TABLE 1

Component	Concentration
Polypeptone (produced by Nihon Pharmaceutical)	1.0%
Yeast extract (produced by Difco)	1.0%
NaCl	0.5%
D-biotin	10 .mu.g/l
Agar	1.5%
pH 7.2	

As a result, it was confirmed that the growth-permitting concentration had a threshold value in the vicinity of 3 mg/dl of PESP concentration at 35.degree. C. as shown in Table 2.

TABLE 2

PESP concentration (mg/dl)	0	0.1	0.3	1.0	3.0	10	30
Colony formation	+	+	+	+	+	-	-

2. Induction of Mutant Strain Exhibiting Temperature Sensitivity to Biotin Action-Suppressing Agent

Brevibacterium lactofermentum ATCC 13869 was cultivated on a bouillon agar medium at 31.5.degree. C. for 24 hours to obtain bacterial cells. The obtained bacterial cells were treated at 30.degree. C. for 30 minutes with an aqueous solution of 250 .mu.g/ml of N-methyl-N'-nitro-N-nitrosoguanidine. A suspension of the bacterial cells at a survival ratio of 1% was then seeded on a CM2B agar plate medium, and cultivated at 31.5.degree. C. for 20-30 hours to form colonies. They were replicated on a CM2B agar medium and a CM2B agar medium added with 3 mg/dl of PESP respectively, and cultivated at 35.degree. C. for 20-30 hours. Bacterial strains were obtained, which grew on the CM2B medium but made no observable growth on the CM2B medium containing 3 mg/dl of PESP. Thus 720 strains were obtained from about 10,000 colonies. Each of the obtained bacterial strains was reconfirmed for the presence or absence of growth at 35.degree. C. on the CM2B agar plate medium containing 3 mg/dl of PESP. Strains which apparently exhibited no sensitivity were excluded, and 435 strains which exhibited sensitivity were obtained.

3. Confirmation of L-Glutamic Acid Productivity of Mutant Strains Exhibiting Temperature Sensitivity to Biotin Action-Suppressing Agent

The L-glutamic acid productivity was confirmed as follows for the 435 mutant strains obtained in the item 2. described above and their parent strain ATCC 13869.

ATCC 13869 strain and each of the mutant strains were cultivated on the CM2B agar medium at 31.5.degree. C. for 20-30 hours respectively to obtain bacterial cells which were seeded in a liquid medium having a composition of Medium A in Table 3 to start cultivation with shaking at 31.5.degree. C. After about 22 hours, a medium was newly added so that final concentrations were shown in Medium B in Table 3, followed by performin- cultivation for about 24 hours at 31.5.degree. C. or after shifting the cultivation temperature to 35.degre . C. or 37.degr e. C.

After completion of the cultivation, the presence or absence of L-glutamic acid production was investigated by using a Biot ch Analyzer produced by Asahi Ch mical Industry. As a result, it was confirmed that 106 strains among the 435 mutant strains produced glutamic acid.

TABLE 3

Component	Medium A	Medium B
Glucose	3 g/dl	5 g/dl
KH.sub.2 PO.sub.4	0.14 g/dl	0.14 g/dl
MgSo.sub.4 .multidot. 7H.sub.2 O	0.04 g/dl	0.04 g/dl
FeSO.sub.4 .multidot. 7H.sub.2 O	0.001 g/dl	0.001 g/dl
MnSO.sub.4 .multidot. 4H.sub.2 O	0.001 g/dl	0.001 g/dl
(NH.sub.4).sub.2 SO.sub.4	1.5 g/dl	2.5 g/dl
Soybean protein hydrolysate solution	1.5 ml/dl	0.38 ml/dl
Thiamin hydrochloride	0.2 mg/l	0.2 mg/l
Biotin	0.3 mg/l	0.3 mg/l
Antifoaming agent	0.05 ml/l	0.05 ml/l
CaCO.sub.3	5 g/dl	5 g/dl
pH 7.0 (adjusted with KOH)		

Accumulation amounts of L-glutamic acid at each temperature are shown in Table 4 for representative strains of the mutant strains and ATCC 13869 strain.

TABLE 4

Accumulation of glutamic acid by mutant strains (g/dl)			
Bacterial strain	Cultivation temperature after temperature shift		
	31.5.degree. C.	35.degree. C.	37.degree. C.
ATCC 13869	0.0	0.0	0.2
No. 21	0.3	2.8	2.9
No. 36	2.4	2.6	2.7
No. 58	0.2	1.9	2.7
No. 100	0.4	1.5	2.8
No. 121	0.5	1.7	1.9

4. Confirmation of Temperature Sensitivity to Biotin Action-Suppressing Agent

Temperature sensitivity to PESP of the mutant strains obtained in the item 3. described above was confirmed as follows by means of liquid culture.

Each of the mutant strains and their parent strain were cultivated on the CM2B agar plat medium at 31.5.degree. C. for 24 hours to obtain bacterial cells. The obtained b cterial cells were inoculated to a CM2B liquid medium and a CM2B liquid medium containing PESP at a concentration of 3 mg/dl to perform cultivati n with shaking at 31.5.degree. C. and 37.degree. C. for 24 hours. Optical densities of obtained culture liquids were measured at 660 nm. The relativ growth degree in the PESP-added medium was determined provided that growth in the medium without addition of PESP at each temperature was regarded as 100. Results are shown in Table 5.

TABLE 5

Bacterial strain	Relative Growth degree 31.5.degree. C.	37.degree. C.
ATCC 13869	98	85
No. 21	81	33
No. 36	52	15
No. 58	85	32
No. 100	82	32
No. 121	95	40

As shown in this table, Brevibacterium lactofermentum ATCC 13869 as a parent strain had a relative growth degree of 85 at 37.degree. C. in the presence of 3 mg/dl of PESP, while each of the mutant strains had a relative growth degree of 40 or less in the presence of PESP, clearly having sensitivity to PESP at a concentration of 3 mg/dl.

The relative growth degree at 31.5.degree. C. and 35.degree. C. in the presence of several concentrations of PESP is shown in FIG. 1 for the mutant strain No. 21 among the mutant strains obtained in the item 3. described above and its parent strain ATCC 13869.

Among the mutant strains, No. 21 has been designated as Brevibacterium lactofermentum AJ13029, deposited on Sep. 2, 1994 in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology under a deposition number of FERM P-14501, transferred to international deposition based on the Budapest Treaty on Aug. 1, 1995, and awarded a deposition number of FERM BP-5189.

EXAMPLE 2

Production of L-Glutamic Acid

1. Investigation on Cultivation Temperature Shift Timing

Brevibacterium lactofermentum ATCC 13869 or AJ13029 was inoculated to a seed culture medium having a composition shown in Table 6, and cultivated with shaking at 31.5.degree. C. for 24 hours to obtain a seed culture. A medium for full-scale cultivation having a composition shown in Table 6 was dispensed into each amount of 300 ml and poured into a jar fermenter made of glass having a volume of 500 ml, and sterilized by heating. After that, 40 ml of the seed culture was inoculated thereto. Cultivation was started by using an agitation speed of 800-1,300 rpm, an aeration amount of 1/2-1/1 vvm, and a cultivation temperature of 31.5.degree. C. The culture liquid was maintained to have pH of 7.5 by using ammonia gas. The cultivation

temperature was shifted to 37.degree. C., 8, 12 or 16 hours after the start of cultivation. A control for comparison was provided in which the cultivation temperature was not shifted, and cultivation was continued xactly at 31.5.degr e. C.

TABLE 6

Component	Concentration Seed culture	Full-scale culture
Glucose	5 g/dl	15 g/dl
KH.sub.2 PO.sub.4	0.1 g/dl	0.2 g/dl
MgSO.sub.4 .multidot. 7H.sub.2 O	0.04 g/dl	0.15 g/dl
FeSO.sub.4 .multidot. 7H.sub.2 O	1 mg/dl	1.5 mg/dl
MnSO.sub.4 .multidot. 4H.sub.2 O	1 mg/dl	1.5 mg/dl
Soybean protein hydrolysate solution	2 ml/dl	6 ml/dl
Biotin	50 .mu.g/l	200 .mu.g/l
Thiamin hydrochloride	200 .mu.g/l	300 .mu.g/l

In any experiment, the cultivation was finished at a point in time of 20–40 hours at which glucose was completely consumed. The amount of L-glutamic acid produced and accumulated in the culture liquid was measured. Results are shown in Table 7.

TABLE 7

Temperature shift timing (hour)	L-Glutamic acid production amount (g/dl) ATCC 13869	AJ13029
8	0.6	8.3
12	0.1	7.0
16	0.0	5.4
--	0.0	2.1

According to the results, it is understood that AJ13029 strain produces L-glutamic acid in the absence of any biotin action-suppressing agent even in the medium containing an excessive amount of biotin by shifting the cultivation temperature from 31.5.degree. C. to 37.degree. C., and that the amount of L-glutamic acid increases in proportion to the earliness of the shift timing of the cultivation temperature. On the contrary, in the case of ATCC 13869 as the parent strain, little production of L-glutamic acid was observed even by shifting the cultivation temperature.

Bacterial cells were removed by centrifugation from 1 L of cultivation-finished culture liquid having been subjected to the temperature shift 8 hours after the start of cultivation. L-glutamic acid was separated and purified from an obtained supernatant in accordance with an ordinary method using an ion exchange resin. Crystals of obtained sodium L-glutamate were 64.3 g.

2. Investigation on Shift Temperature

Cultivation of Brevibacterium lactofermentum ATCC 13869 and AJ13029 was started at 31.5.degree. C. by using a jar fermenter made of glass having a volume of 500 ml in the same manner as the item 1. described above. The cultivation temperature was shifted to 34.degree. C., 37.degree. C. or 39.degree. C., 8 hours after the start of cultivation. A control for comparison was provided in which the cultivation temperature was not shifted to continue cultivation exactly at 31.5.degree. C. In any experiment, the cultivation was finished at a point in time of 20–40 hours at which glucose was completely consumed. The amount of L-glutamic acid produced and accumulated in the culture liquid was measured. Results are shown in Table 8.

TABLE 8

Temperature after shift (.degree.C.)	L-glutamic acid production amount (g/dl)	
	ATCC 13869	AJ13029
34	0.0	5.8
37	0.5	8.3
39	0.9	9.2
—	0.0	2.1

According to the results, it is understood that there is a tendency that the amount of L-glutamic acid increases as the temperature after the shift becomes high when AJ13029 strain is cultivated and the cultivation temperature is shifted.

EXAMPLE 3

Preparation of Temperature-Sensitive Mutant Strain to Biotin Action-Suppressing Agent Originating from Coryneform L-Glutamic Acid-Producing Bacterium by Genetic Recombination

1. Preparation of Chromosomal DNA of Brevibacterium lactofermentum ATCC 13869 (Wild Strain of Coryneform L-Glutamic Acid-Producing Bacterium)

Brevibacterium lactofermentum ATCC 13869 was inoculated to 100 ml of T-Y medium (Bacto-tryptone (Difco) 1%, Bacto-yeast extract (Difco) 0.5%, NaCl 0.5% (pH 7.2)), and cultivated at a temperature of 31.5.degree. C. for 8 hours to obtain a culture preparation. The culture preparation was treated by centrifugation at 3,000 r.p.m. for 15 minutes to obtain 0.5 g of wet bacterial cells. Chromosomal DNA was then obtained from the bacterial cells in accordance with a method of Saito and Miura (Biochem. Biophys. Acta., 72, 619 (1963)). Next, 60 .mu.g of the chromosomal DNA and 3 units of a restriction enzyme Sau3AI were respectively mixed with 10 mM Tris-HCl buffer (containing 50 mM NaCl, 10 mM MgSO.sub.4, and 1 mM dithiothreitol (pH 7.4)), and reacted at a temperature of 37.degree. C. for 30 minutes. The solution after completion of the reaction was subjected to treatments of phenol extraction and ethanol precipitation in accordance with an ordinary procedure to obtain 50 .mu.g of chromosomal DNA fragments of Brevibacterium lactofermentum ATCC 13869 digested with Sau3AI.

2. Construction of Gene Library of Brevibacterium lactofermentum ATCC 13869 by Utilizing Plasmid Vector DNA

In order to prepare a gene library capable of introduction into cells of both Escherichia coli and bacteria belonging to the genus Corynebacterium, a plasmid autonomously replicable in cells of the both was prepared. Specifically, a plasmid pHK4 (Japanese Patent Laid-open N . 5-7491) having a replication origin from an already obtained plasmid pHM1519 (Agric. Biol. Chem., 48, 2901-2903 (1984)) autonomously replicable in coryneform bacteria was digested with restriction enzymes BamHI and KpnI to obtain a gene fragment containing the replication origin. The obtained fragment was blunt-ended by using a DNA blunt end formation kit (produced by Takara Shuzo, Blunting kit), and then inserted into a SalI site of a plasmid vector pHSG399 (produced by Takara Shuzo) by using a SalI linker (produced by Takara Shuzo) to construct pSAC4. Escherichia coli HB101 harboring pHK4 has been designated as Escherichia coli AJ13136, and deposited on Aug. 1, 1995 in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology under a deposition number of FERM BP-5186.

pSAC4 (20 .mu.g) constructed as described above and a restriction enzyme BamHI (20 units) were mixed with 50 mM Tris-HCl buffer (containing 100 mM NaCl and 10 mM magnesium sulfate (pH 7.4)), and reacted at a temperature of 37.degree. C. for 2 hours to obtain a digested solution. The solution was subjected to treatments of phenol extraction and ethanol precipitation in accordance with an ordinary procedure. After that, in order to prevent DNA fragments originating from the plasmid vector from religation, the DNA fragments were dephosphorylated by means of a treatment of bacterial alkaline phosphatase in accordance with a method of Molecular Cloning 2nd edition (J. Sambrook, E. F. Fritsch and T. Maniatis, Cold Spring Harbor Laboratory Press, pl. 56 (1989)), followed by treatments of phenol extraction and ethanol precipitation in accordance with an ordinary procedure.

pSAC4 (1 .mu.g) digested with BamHI, the chromosomal DNA fragments (1 .mu.g) of Brevibacterium lactofermentum ATCC 13869 digested with Sau3AI having been obtained in the item 1., and T4 DNA ligase (2 units) (produced by Takara Shuzo) were added to 66 mM Tris-HCl buffer (pH 7.5) containing 66 mM magnesium chloride, 10 mM dithiothreitol, and 10 mM ATP, and reacted at a temperature of 16.degree. C. for 16 hours to ligate DNA. Next, the DNA mixture was used to transform Escherichia coli DH5 in accordance with an ordinary method, which was spread on an L-agar medium containing 170 .mu.g/ml of chloramphenicol. About 20,000 colonies were obtained. Thus a gene library was constructed.

3. Transformation of Brevibacterium lactofermentum AJ11060

Recombinant DNA was recovered from the about 20,000 colonies described above. The recovery was performed in accordance with the method of Saito and Miura described above.

The recombinant DNA mixture was divided into 50 batches which were introduced into the mutant strain AJ11060 having increased surfactant sensitivity in accordance with an ordinary method for transformation by using electric pulses (Japanese Patent Laid-open No. 2-207791). Transformants were inoculated to a glucose-added L-agar medium, and stationarily cultivated at 31.5.degree. C. Thus about 20,000 transformants appeared. These transformants were then replicated on the plate containing 30 mg/l of a surfactant. Several strains, which exhibited resistance to the surfactant and were capable of growth on the aforementioned plate, were obtained from them.

4. Examination of Surfactant Resistance of Strain Harboring Multiple Copies of dtsR Gene

Recombinant DNA was extracted respectively from grown several strains, and AJ11060 strain was retransformed by using the DNA. A strain which exhibited surfactant resistance also in this experiment was obtained. Recombinant DNA harbored by this strain was designated as "pDTR6", and the gene to give surfactant resistance carried by the plasmid was designated as "dtsR". AJ11060 strain into which the plasmid has been introduced was suppressed in growth inhibition

in a liquid medium (80 g of glucose, 1 g of KH.sub.2 PO.sub.4, 0.4 g of MgSO.sub.4.7H.sub.2 O, 30 g of (NH.sub.4).sub.2 SO.sub.4, 0.01 g of FeSO.sub.4.7H.sub.2 O, 0.01 g of MnSO.sub.4.7H.sub.2 O, 15 ml of soybean hydrolysate, 200 .mu.g of thiamine hydrochloride, 300 .mu.g of biotin, 4 mg of chloramphenicol, 3.0 g of polyoxyethylene sorbitan monopalmitate and 50 g of CaCO.sub.3 in one liter of pure water (the pH of the medium having been adjusted to 8.0 with KOH)) to which 3 g/L of a surfactant was added (FIG. 2).

5. Preparation of Plasmid DNA

The plasmid was prepared in accordance with an ordinary method from AJ11060/pDTR6 containing the recombinant DNA obtained as described above, and introduced into Escherichia coli JM109. Obtained Escherichia coli JM109/pDTR6 was preliminarily cultivated at a temperature of 37.degree. C. for 24 hours in 20 ml of a medium comprising tryptone 1%, yeast extract 0.5%, and NaCl 0.5%. An obtained culture liquid (20 ml) was inoculated to a medium (1 l) having the same composition as that described above to perform cultivation at a temperature of 37.degree. C. for 3 hours, followed by addition of chloramphenicol (0.2 g). Cultivation was further performed at the same temperature for 20 hours to obtain a culture liquid. Next, the culture liquid was treated by centrifugation at 3,000 r.p.m. for 10 minutes to obtain each 2 g of wet bacterial cells which were suspended in 350 mM Tris-HCl buffer (20 ml, pH 8.0) containing 25% sucrose. Lysozyme (produced by Sigma) 10 mg, 0.25M EDTA solution (8 ml, pH 8.0), and 20% sodium dodecyl sulfate solution (8 ml) were then respectively added thereto. A temperature-holding treatment was performed at a temperature of 60.degree. C. for 30 minutes to obtain a bacterial lysate solution. A solution of 5M NaCl (13 ml) was added to the bacterial lysate solution to perform a treatment at a temperature of 4.degree. C. for 16 hours, followed by centrifugation at 15,000 r.p.m. for 30 minutes. An obtained supernatant was subjected to treatments of phenol extraction and ethanol precipitation in accordance with an ordinary procedure to precipitate DNA.

The precipitate was dried under a reduced pressure, and then dissolved in 10 mM Tris-HCl buffer (6 ml, pH 7.5) containing 1 mM EDTA. Cesium chloride (6 g) and ethidium bromide (0.2 ml, 19 mg/ml) were added thereto. An equilibrium density-gradient centrifugation was performed at 39,000 r.p.m. for 42 hours by using an ultracentrifuge to isolate DNA. Ethidium bromide was removed by using n-butanol, followed by dialysis against 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA to obtain about 500 .mu.g of pDTR6 as purified recombinant DNA. A private number of AJ12967 is given to Escherichia coli JM109/pDTR6. This strain has been deposited on Feb. 22, 1994 in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology under a deposition number of FERM P-14168, transferred to international deposition based on the Budapest Treaty on Feb. 9, 1995, and awarded a deposition number of FERM BP-4994.

6. Analysis of Nucleotide Sequence of DNA Containing dtsR Gene

The nucleotide sequence was determined by using the recombinant DNA obtained in the item 5. The nucleotide sequence was determined in accordance with a method of Sanger by using Taq DyeDeoxy Terminator Cycle Sequencing Kit (produced by Applied Biochemical). The obtained DNA containing the dtsR gene had a nucleotide sequence as shown in SEQ ID NO: 1 in Sequence Listing. The longest open reading frame existing in this sequence was a nucleotide sequence from 359th A to 1987th G in the nucleotide sequence shown in SEQ ID NO: 1. However, it was postulated that 467-469th ATG might be an initiation codon according to analysis of a consensus sequence existing in a region upstream from the gene. An amino acid sequence, which can be encoded by the open reading frame from 359th A to 1987th G, is shown in SEQ ID NO: 1 in Sequence Listing together with its nucleotide sequence. The amino acid sequence is singly shown in SEQ ID NO: 2 in Sequence Listing. A protein encoded by a nucleotide sequence comprising 467-1987th nucleotides was regarded as "DTSR protein".

It is well-known that the methionine residue existing at the N-terminal of a protein is removed by the action of peptidas after translation. This is due to the fact that the methionine at the N-terminal originates from ATG as the translation initiation codon, and thus it often has no relation to an essential function of the protein. It is probable that the removal of methionine residue may occur also in the case of the DTSR protein of the present invention.

The nucleotide sequence and the amino acid sequence were respectively compared with known sequences with respect to their homology. EMBL and SWISS-PROT were used as data bases. As a result, it has been confirmed that the gene shown in SEQ ID NO: 1 in Sequence Listing and the protein encoded by it are novel. However, it has been revealed that there is homology to an already reported protein. This protein is described as .beta.-subunit of propionyl-CoA carboxylase (PPC) protein in Proc. Natl. Acad. Sci. USA, vol. 83 (1986) 8049-8053; Proc. Natl. Acad. Sci. USA, vol. 83 (1986) 4864-4868; and Gene, vol. 122 (1992) 199-202.

7. Preparation of Mutant Type dtsR Gene

The dtsR gene coding for the temperature-sensitive mutant type DTSR protein was obtained in accordance with the following method. pDTR6 plasmid was subjected to a hydroxylamine treatment in vitro in accordance with a method described in a literature, Shortle, D. and Nathans, D., Proc. Natl. Acad. Sci. U.S.A., 75, 270 (1978), and it was introduced into AJ11060 by using the electric pulse method described above. About 20,000 strains of transformants were cultivated on an M-CM2G agar medium at 25.degree. C. for 30 hours to form colonies. The colonies on each plate were replicated on two plates of the medium containing 30 mg/l of a surfactant, followed by cultivation at 31.5.degree. C. and 35.degree. C. for 20 hours. After that, two strains were obtained which grew at 31.5.degree. C. but did not grow at 35.degree. C. Plasmids were extracted from the two strains in accordance with an ordinary method. Thus pDTR6-11 and pDTR6-77 were obtained.

8. Construction of Mutant Type dtsR Gene-Introduced Strains by Gene Substitution

Mutant type dtsR gene-substituted strains were obtained in accordance with a homologous recombination method by using a temperature-sensitive plasmid as described in Japanese Patent Laid-open 5-7491. Specifically, pDTR6-11 and pDTR6-77 described above were digested with XbaI and KpnI, and the obtained fragments containing the dtsR gene thereof were ligated with pHSG398 (produced by Takara Shuzo) having been digested with XbaI and KpnI by using the method described above to obtain pHSGX-K-11 and pHSGX-K-77 respectively.

Next, a plasmid pHSC4 (Japanese Patent Laid-open No. 5-7491) having a mutant type replication origin with its temperature-sensitive autonomous replicability obtained from a plasmid autonomously replicable in coryneform bacteria was digested with restriction enzymes BamHI and KpnI to obtain a gene fragment containing the replication origin. The obtained DNA fragment was blunt-ended by using a DNA blunt end formation kit (produced by Takara Shuzo, Blunting kit), and then inserted into KpnI recognition sites of pHSGX-K-11 and pHSGX-K-77 by using a KpnI linker (produced by Takara Shuzo) to construct plasmids pKTCX-K-11 and pKTCX-K-77. Escherichia coli AJ12571 harboring pHSC4 has been deposited on Oct. 11, 1990 in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology under a deposition number of FERM P-11763, transferred to international deposition based on the Budapest Treaty on Aug. 26, 1991, and deposited under a deposition number of FERM BP-3524.

The two plasmids were respectively introduced into Brevibacterium lactofermentum ATCC 13869 by using an electric pulse method, and the dtsR gene on chromosome was substituted into a mutant type in accordance with a method described in Japanese Patent Laid-op n No. 5-

7491. Specifically, Brevibacterium lactofermentum ATCC 13869/pKTCX-K-11 and ATCC 13869/pKTCX-K-77 were cultivated with shaking in an M-CM2G liquid medium at 25.degree. C. for 6 hours, and then spread on an M-CM2G medium containing 5 .mu.g/ml f chloramphenicol. Strains which formed colonies at 34.degree. C. were obtained as plasmid-incorporated strains. Next, strains, which were sensitive to chloramphenicol at 34.degree. C., were obtained from the plasmid-incorporated strains by using a replica method. No. 11 strain and No. 77 strain as strains with lost surfactant resistance at 34.degree. C. were obtained from the sensitive strains. In these strains, the dtsR gene on chromosome is substituted into a mutant type. A DNA fragment obtained by amplifying the region containing the mutant type dtsR gene of the strain No.11 by PCR method using the plasmid pHSGX-K-11 as a template was inserted at HincII recognition site of the plasmid pHSG299 (produced by Takara Shuzo) to obtain a plasmid pHSGDTSR11. Escherichia coli JM109 into which the plasmid pHSGDTSR11 was introduced was designated as AJ13137, and deposited on Aug. 1, 1995 in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology under a deposition number of FERM BP-5187. The mutant type dtsR gene of the strain No.11 can be obtained by digesting the plasmid pHSGDTSR11 with the restriction enzymes SphI and KpnI.

9. L-Glutamic Acid Productivity of No. 11 Strain and No. 77 Strain

The productivity of L-glutamic acid was evaluated for No. 11 strain and No. 77 strain obtained in the item 8. described above in the same manner as Example 2. Specifically, the same media as those in Example 2 were used, and the cultivation temperature was shifted to 37.degree. C. on the 8th hour after the start of cultivation. As a result, the yield of L-glutamic acid of the strains having the mutant type gene was improved as shown in Table 9.

TABLE 9

Bacterial strain	L-Glutamic acid (g/dl)
ATCC 13869	0.5
No. 11	7.5
No. 77	6.9

EXAMPLE 4

Enhancement of Genes of Glutamic Acid Biosynthesis System in Surfactant Temperature-Sensitive Mutant Strain Originating from L-Glutamic Acid-Producing Bacterium

1. Cloning of gdh, gltA and icd Genes

gdh (glutamate dehydrogenase gene), gltA (citrate synthase gene), and icd (isocitrate dehydrogenase gene) of Brevibacterium lactofermentum were cloned by the PCR method. Primers to be used for the PCR method were synthesized on the basis of already reported sequences of gdh gene (Molecular Microbiology, 6(3), 317-326 (1992)), gltA gene (Microbiology, 140, 1817-1828 (1994)), and icd gene (J. Bacteriol., 177, 774-782 (1995)) of Corynebacterium glutamicum. Oligonucleotides shown in SEQ ID NO: 3 (5' side) and SEQ ID NO: 4 (3' side) in Sequence Listing as primers for amplifying the gdh gene, oligonucleotides shown in SEQ ID NO: 5 (5' side) and SEQ ID NO: 6 (3' side) as primers for amplifying the gltA gene, and oligonucleotides shown in SEQ ID NO: 7 (5' side) and SEQ ID NO: 8 (3' side) as primers for amplifying the icd gene were respectively synthesized and used.

Chromosomal DNA was prepared from Brevibacterium lactofermentum ATCC13869 in accordance with the method described in Exampl 3, which was used as a template to perform PCR by using the aforementioned oligonucleotides as primers. Both ends of obtained amplification products were blunt-ended by using a commercially available DNA blunt end formation kit (produced by Takara Shuzo, Blunting kit), followed by cloning into a SmaI site of a vector plasmid pHSG399 (produced by Takara Shuzo) respectively to obtain plasmids pHSG-gdh, pHSG-gltA, and pHSG-icd.

2. Cloning of ppc Gene

Chromosomal DNA of Brevibacterium lactofermentum ATCC13869 was prepared in accordance with the method described in Example 3, which was used as a template to obtain a DNA fragment of about 3.4 Kbp containing ppc gene coding for PEPC (phosphoenolpyruvate carboxylase) by using the PCR method. Primers to be used for the PCR method was synthesized on the basis of an already reported sequence of ppc gene of Corynebacterium glutamicum (Gene, 77, 237-251 (1989)). The PCR reaction was conducted in the same manner as described above. Sequences of the primers are shown in SEQ ID NO: 9 (5' side) and SEQ ID NO: 10 (3' side).

An amplification product of the PCR reaction was digested with a restriction enzyme SalI (produced by Takara Shuzo) and inserted at a SalI site of the plasmid pHSG399 to obtain a plasmid pHSG-ppc'. The ppc gene of pHSG-ppc' is inserted in a direction opposite to that of lac promoter of pHSG399.

Next, a promoter of tryptophan operon known as a promoter capable of operation in Brevibacterium lactofermentum (Gene, 53, 191-200 (1987)) was inserted into a position upstream from the ppc gene on pHSG-ppc'. This promoter is known to exhibit its activity with a sequence comprising 51 nucleotides shown in SEQ ID NO: 11 in Sequence Listing. A nucleotide strand having the sequence shown in SEQ ID NO: 11 and a nucleotide strand having a sequence of SEQ ID NO: 12 as a complementary strand of it were synthesized so as to obtain double-strand DNA containing the 51 base pairs having the promoter activity with both ends coinciding with those of a digested fragment by restriction enzymes KpnI and XbaI.

The both synthesized DNA's were mixed at a concentration of about 10 pmol/.mu.l for each of them, heated at 100.degree. C. for 10 minutes, and then left and cooled at room temperature t achieve annealing. pHSG-ppc' was digested with restriction enzymes KpnI and XbaI (produced by Takara Shuzo), and ligated with the aforementioned promoter. The ligation reaction was performed by using a ligation kit produced by Takara Shuzo. Thus a plasmid pHSG-ppc was obtained in which one copy of the promoter of tryptophan operon was inserted into a position upstream from the ppc gene.

3. Construction of Plasmid Incorporated with Three Types of Genes of gdh, gltA, and icd

Three types of the genes of gdh, gltA, and icd were ligated to construct a plasmid. Specifically, the plasmid pHSG-gdh was digested with a restriction enzyme EcoRI, and blunt-ended by using a commercially available DNA blunt end formation kit (produced by Takara Shuzo, Blunting kit), with which the PCR-amplified product of the gltA gene with both ends blunt-ended as described above was ligated to obtain a plasmid pHSG-gdh+gltA. Further, the plasmid pHSG-gdh+gltA was digested with a restriction enzyme KpnI, and blunt-ended in the same manner, with which the PCR-amplified product of the icd gene with both ends blunt-ended as described above was ligated to obtain a plasmid pHSG-gdh+gltA+icd.

4. Construction of Plasmid Incorporated with Three Types of Genes of gdh, gltA, and ppc

Three types of the genes of gdh, gltA, and ppc were ligated to construct a plasmid. Specifically, th plasmid pHSG-gdh+gltA was digested with a restriction enzyme KpnI. The plasmid pHSG-ppc was digested with restriction enzymes KpnI and SalI to obtain a ppc gene fragment having the promoter of tryptophan operon at an upstream position. The obtained fragment was blunt-ended by using a DNA blunt end formation kit (produced by Takara Shuzo, Blunting kit), and then inserted into a KpnI site of the plasmid pHSG-gdh+gltA by using a KpnI linker (produced by Takara Shuzo) to obtain a plasmid pHSG-gdh+gltA+ppc.

5. Introduction of Replication Origin for Coryneform Bacterium into the Aforementioned Plasmids

In order to make pHSG-gdh, pHSG-gltA, pHSG-ppc, pHSG-icd, pHSG-gdh+gltA+icd, and pHSG-gdh+gltA+ppc autonomously replicable in cells of coryneform bacteria, an already obtained replication origin originating from a plasmid pHM1519 (Agric. Biol. Chem., 48, 2901-2903 (1984)) autonomously replicable in coryneform bacteria was introduced into pHSG-gdh, pHSG-gltA, pHSG-ppc, pHSG-icd, pHSG-gdh+gltA+icd, and pHSG-gdh+gltA+ppc.

Specifically, a plasmid pHK4 (Japanese Patent Laid-open No. 5-7491) having the replication origin from pHM1519 was digested with restriction enzymes BamHI and KpnI to obtain a gene fragment containing the replication origin. The obtained fragment was blunt-ended by using a DNA blunt end formation kit (produced by Takara Shuzo, Blunting kit), and then inserted into a KpnI site of pHSG-gdh, pHSG-gltA, pHSG-ppc, and pHSG-icd respectively by using a KpnI link r (produced by Takara Shuzo) to obtain pGDH, pGLTA, pPPC, and pICD. The replication origin from pHM1519 was inserted into pHSG-gdh+gltA+icd and pHSG-gdh+gltA+ppc respectively at their SalI sites in the same manner by using the SalI linker (produced by Takara Shuzo) to obtain pGDH+GLTA+ICD and pGDH+GLTA+PPC.

6. Confirmation of Expression of Each of Genes Contained in pGDH, pGLTA, pPPC, pICD, pGDH+GLTA+ICD, and pGDH+GLTA+PPC

It was confirmed that each of the genes on pGDH, pGLTA, pPPC, pICD, pGDH+GLTA+ICD, and pGDH+GLTA+PPC was expressed in cells of Brevibacterium lactofermentum, and that these plasmids fulfilled the function of gene amplification.

Specifically, each of the plasmids was introduced into Brevibacterium lactofermentum ATCC 13869 by using an electric pulse method (Japanese Patent Laid-open No. 2-207791). Obtained transformants were selected on a CM2G plate medium containing 4 .mu.g/ml of chloramphenicol (containing polypeptone 10 g, yeast extract 10 g, glucose 5 g, NaCl 5 g, and agar 15 g per 1 L of pure water, pH 7.2). The obtained transformants were cultivated on the CM2G agar medium, inoculated to a medium containing glucose 80 g, KH.sub.2 PO.sub.4 1 g, MgSO.sub.4.7H.sub.2 O 0.4 g, (NH.sub.4).sub.2 SO.sub.4 30 g, FeSO.sub.4.7H.sub.2 O 0.01 g, MnSO.sub.4.7H.sub.2 O 0.01 g, soybean hydrolysate solution 15 ml, thiamin hydrochloride 200 .mu.g, biotin 300 .mu.g, and CaCO.sub.3 50 g per 1 L of pure water (with pH adjusted to 8.0 with KOH), and cultivated at 31.5.degree. C. for 16 hours. The culture liquid was centrifuged in accordance with an ordinary method to collect bacterial cells.

Crude extracts obtained by disrupting the bacterial cells were used to measure the GDH (glutamate dehydrogenase) activity of ATCC 13869/pGDH, ATCC 13869/pGDH+GLTA+ICD, and ATCC 13869/pGDH+GLTA+PPC in accordance with a method described in Molecular Microbiology, 6(3), 317-326 (1992). As a result, it was found that each of the transformants had the GDH activity which was about 13 times greater than that of ATCC 13869/pSAC4 as a control (Table 10).

The CS (citrate synthase) activity of ATCC 13869/pGLTA, ATCC 13869/pGDH+GLTA+ICD, and

ATCC 13869/pGDH+GLTA+PPC was measured in accordance with a method described in Microbiology, 140, 1817–1828 (1994). The ICDH (isocitrate dehydrogenase) activity of ATCC 13869/pICD and ATCC 13869/pGDH+GLTA+ICD was measured in accordance with a method described in J. Bacteriol., 177, 774–782 (1995). The PEPC activity of ATCC 13869/pPPC and ATCC 13869/pGDH+GLTA+PPC was measured in accordance with a method described in Gene, 77, 237–251 (1989). Measurement results are shown in Tables 11–13. It was found that any transformant had the activity of each of the objective enzymes which was about 2–20 times greater than that of ATCC 13869/pSAC4 as a control. According to this fact, it was confirmed that each of the genes on pGDH, pGLTA, pPPC, pICD, pGDH+GLTA+ICD, and pGDH+GLTA+PPC was expressed in cells of Brevibacterium lactofermentum, and fulfilled the function thereof.

TABLE 10

Bacterial strain	GDH activity (.increment.Abs/min/mg protein)
ATCC 13869/pGDH	1.36
ATCC 13869/PGDH + GLTA + ICD	1.28
ATCC 13869/pGDH + GLTA + PPC	1.33
ATCC 13869/pSAC4	0.11

TABLE 11

Bacterial strain	CS activity (.mu.mol/min/mg protein)
ATCC 13869/pGLTA	5.5
ATCC 13869/pGDH + GLTA + ICD	4.8
ATCC 13869/pGDH + GLTA + PPC	4.8
ATCC 13869/pSAC4	0.7

TABLE 12

Bacterial strain	PEPC activity (Units/min/mg protein)
ATCC 13869/pPPC	1.12
ATCC 13869/pGDH + GLTA + PPC	1.04
ATCC 13869/pSAC4	0.11

TABLE 13

Bacterial strain	ICDH activity (Units/min/mg protein)
ATCC 13869/pICD	3.5
ATCC 13869/pGDH + GLTA + ICD	2.8
ATCC 13869/pSAC4	1.0

7. L-Glutamic Acid Production by AJ13029 Strain, and AJ13029 Strains with Amplified gdh, gltA, ppc, and icd Genes

A medium (300 ml) containing glucose 60 g, KH.sub.2 PO.sub.4 1 g, MgSO.sub.4.7H.sub.2 O 0.4 g, (NH.sub.4).sub.2 SO.sub.4 30 g, FeSO.sub.4.7H.sub.2 O 0.01 g, MnSO.sub.4.7H.sub.2 O 0.01 g, soybean hydrolysate solution 15 ml, thiamin hydrochloride 200 .mu.g, and biotin 450 .mu.g per 1 L of pure water was poured into a jar fermenter having a volume of 1 l, and sterilized by heating. Bacterial cells of each of the strains obtained by cultivation on a CM2G agar medium were inoculated thereto, and cultivated at 31.5.degree. C. for 30 hours while controlling pH at 7.0 with ammonia gas.

The bacterial cell density and the amount of L-glutamic acid accumulated in the medium after the cultivation were measured in the same manner as described above. Results are shown in Table 14.

TABLE 14

Bacterial strain	Plasmid	Cell density (OD)	L-glutamic acid (g/l)
AJ13029	--	0.95	33
AJ13029	pGDH	1.01	35
AJ13029	pGLTA	0.93	37
AJ13029	pICD	0.93	37
AJ13029	pPPC	0.84	38
AJ13029	pGDH + GLTA + ICD	1.05	39
AJ13029	pGDH + GLTA + PPC	0.95	41
AJ13029	pSAC4	0.93	33

EXAMPLE 5

Preparation of Mutant Strains Temperature-Sensitive to Biotin Action-Suppressing Agent from L-Lysin-Producing Strain Originating from Coryneform L-Glutamic Acid-Producing Bacterium

1. Measurement of Sensitivity to Biotin Action-Suppressing Agent of L-Lysine-Producing Strain by Replica Method

Sensitivity of an L-lysine-producing strain having AEC resistance, Brevibacterium lactofermentum AJ11446 (Japanese Patent Publication No. 62-24073) induced by mutation from Brevibacterium lactofermentum ATCC 13869 to polyoxyethylene sorbitan monopalmitate (PESP) was measured as follows in accordance with a replica method.

Brevibacterium lactofermentum AJ11446 was cultivated overnight at 31.5.degree. C. on an MCM2G agar plate medium having a composition shown in Table 15 to obtain bacterial cells. They were suspended in sterilized physiological saline, seeded on the aforementioned agar plate medium, and cultivated at 31.5.degree. C. for 20-30 hours to form colonies. They were replicated on an MCM2G agar medium added with each concentration of PESP, and cultivated at 34.degree. C. for 20-30 hours to observe the growth state.

TABLE 15

Component	Concentration

Glucose	0.5%
Polypeptone (produced by Nihon Pharmaceutical)	1.0%
Yeast extract (produced by Difco)	1.0%
NaCl	0.5%
DL-methionine	0.02%
Agar	1.5%
pH 7.2	

As a result, it was confirmed that the growth had a threshold value in the vicinity of 3 mg/dl of PESP concentration under this condition as shown in Table 16.

TABLE 16

PESP concentration (mg/dl)	0	0.1	0.3	1.0	3.0	10	30
Growth	+	+	+	+	+	-	-

2. Induction of Mutant Strain Exhibiting Temperature Sensitivity to Biotin Action-Suppressing Agent

Brevibacterium lactofermentum AJ11446 was cultivated on a bouillon agar medium at 31.5.degree. C. for 24 hours to obtain bacterial cells. The obtained bacterial cells were treated at 30.degree. C. for 30 minutes with an aqueous solution of 250 .mu.g/ml of N-methyl-N'-nitro-N-nitrosoguanidine. A suspension of the bacterial cells at a survival ratio of 1% was then seeded on an MCM2G agar plate medium, and cultivated at 31.5.degree. C. for 20-30 hours to form colonies. They were replicated on an MCM2G agar medium and an MCM2G agar medium added with 3 mg/dl of PESP respectively, and cultivated at 34.degree. C. for 20-30 hours. Bacterial strains were collected, which grew on the MCM2G medium but made no observable growth on the MCM2G medium containing 3 mg/dl of PESP. Thus 250 strains were obtained from about 10,000 colonies. Each of the obtained bacterial strains was reconfirmed for the presence or absence of growth at 34.degree. C. on the MCM2G agar plate medium containing 3 mg/dl of PESP. Strains which apparently exhibited no sensitivity were excluded, and 166 strains which exhibited temperature sensitivity were obtained.

3. Confirmation of Co-Productivity of L-Lysine and L-Glutamic Acid of Mutant Strains Exhibiting Temperature Sensitivity to Biotin Action-Suppressing Agent

The productivity of L-lysine and L-glutamic acid was confirmed as follows for the 166 mutant strains obtained in the item 2. described above and their parent strain AJ11446.

AJ11446 strain and each of the mutant strains were cultivated on the MCM2G agar medium at 31.5.degree. C. for 20-30 hours respectively to obtain bacterial cells which were seeded in a liquid medium having a composition shown in Table 17 to start cultivation with shaking at 31.5.degree. C. After 16 hours, the cultivation temperature was shifted to 34.degree. C. to perform cultivation exactly for 48 hours in total. After completion of the cultivation, the presence or absence of L-lysine and L-glutamic acid production was investigated by using thin layer chromatography. As a result, it was confirmed that 31 strains among the 166 mutant strains

simultaneously produced the two amino acids. When the 31 strains were cultivated at 31.5.degree. C. for 48 hours without shifting the cultivation temperature, co-producti n of L-lysine and L-glutamic acid was observed with respect to 3 strains.

TABLE 17

Component	Concentration
Glucose	10 g/dl
KH.sub.2 PO.sub.4	0.1 g/dl
MgSO.sub.4 .multidot. 7H.sub.2 O	0.04 g/dl
FeSO.sub.4 .multidot. 7H.sub.2 O	0.001 g/dl
MnSO.sub.4 .multidot. 4H.sub.2 O	0.001 g/dl
(NH.sub.4).sub.2 SO.sub.4	2 g/dl
Soybean protein hydrolysate solution	3 ml/dl
DL-alanine	0.35 g/dl
Nicotinic acid amide	5 mg/l
Thiamin hydrochloride	0.2 mg/l
Biotin	0.3 mg/l
Antifoaming agent	0.05 ml/l
CaCO.sub.3	5 g/dl

pH 7.0

Accumulation amounts of L-lysine and L-glutamic acid are shown in Table 18 for representative strains of the mutant strains and AJ11446 strain.

TABLE 18

Bacterial strain	Temperature shift	Lys (g/dl)	Glu (g/dl)
AJ11446	yes	2.09	0.00
	no	2.41	0.00
EK-015	yes	2.17	0.70
	no	2.35	0.00
EK-036	yes	2.35	0.34
	no	2.20	0.14
EK-100	yes	1.69	0.93
	no	1.71	0.47
EK-112	yes	1.96	0.69
	no	2.50	0.00
EK-117	yes	0.99	1.88
	no	1.70	0.00

4. Confirmation of Temperature Sensitivity to Biotin Action-Suppressing Agent

Temperature sensitivity to PESP of the bacteria for simultaneously producing L-lysine and L-glutamic acid obtained in the item 3. described above was confirmed as follows by means of liquid culture.

Each of the mutant strains and their parent strain were cultivated on the MCM2G agar plate medium at 31.5.degree. C. for 24 hours to obtain bacterial cells. The obtained bacterial cells were inoculated to an MCM2G liquid m dium and an MCM2G liquid medium containing PESP at a concentration of 1 mg/dl to perform cultivation with shaking at 31.5.degree. C. and 34.degree. C. for 24 hours. Optical densities (O.D.) of obtained culture liquids were measured at 660 nm. The relative growth degree in the PESP-added medium was determined provided that growth in the medium without addition of PESP was regarded as 100. Results are shown in Table 19.

TABLE 19

Bacterial strain	Relative growth degree	
	31.5.degree. C.	34.degree. C.
AJ11446	95	90
EK-015	90	27
EK-036	84	45
EK-100	87	22
EK-112	98	36
EK-117	84	47

As shown in this table, each of the mutant strains had a relative growth degree of 80 or more at 31.5.degree. C., but 50 or less at 34.degree. C. in the presence of 1 mg/dl of PESP, clearly having sensitivity to PESP.

Influences exerted by PESP on growth at 31.5.degree. C. and 34.degree. C. are shown in FIG. 3 for EK-112 among the mutant strains obtained in the item 3. described above. Growth approximately equivalent to that in the absence of PESP was observed in the presence of PESP at a concentration of not more than 1 mg/dl at 31.5.degree. C. However, growth in the presence of 1 mg/dl of PESP was remarkably inhibited at 34.degree. C. as compared with growth in the absence of PESP.

Thus it is demonstrated that this mutant strain has temperature sensitivity.

Among the mutant strains, EK-112 has been designated as Brevibacterium lactofermentum AJ12993, deposited on Jun. 3, 1994 in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology under a deposition number of FERM P-14348, transferred to international deposition based on the Budapest Treaty on Aug. 1, 1995, and awarded a deposition number of FERM BP-5188.

EXAMPLE 6

Production of L-Lysine and L-Glutamic Acid by Co-fermentation

1. Investigation on Cultivation Temperature Shift Timing

Brevibacterium lactofermentum AJ11446 or AJ12993 was inoculated to the seed culture medium having the composition shown in Table 6 described above, and cultivated with shaking at 31.5.degree. C. for 24 hours to obtain a seed culture. The medium for full-scale cultivation having the composition shown in Table 6 was dispensed into each amount of 300 ml and poured into a jar fermenter made of glass having a volume of 500 ml, and sterilized by heating. After that, 40 ml of the seed culture was inoculated thereto. Cultivation was started by using an agitation speed

of 800–1,300 rpm, an aeration amount of 1/2–1/1 vvm, and a cultivation temperatur of 31.5.degree. C. The culture liquid was maintained t have pH of 7.5 by using ammonia gas. The cultivation temperature was shifted to 34.degree. C., 8, 12 or 16 hours after the start of cultivation. A control for comparison was provided in which the cultivation temperature was not shifted, and cultivation was continued exactly at 31.5.degree. C.

In any experiment, the cultivation was finished at a point in time of 40–50 hours at which glucose was completely consumed. The amounts of L-lysine and L-glutamic acid produced and accumulated in the culture liquid were measured. Results are shown in Table 20.

TABLE 20

	AJ11446		AJ12993	
Temperature shift timing (hour)	Lys (g/dl)	Glu (g/dl)	Lys (g/dl)	Glu (g/dl)
8	5.4	0.0	4.4	1.8
12	5.5	0.0	4.5	1.6
16	5.6	0.0	4.7	1.2
—	5.9	0.0	6.0	0.0

According to the results, it is understood that AJ12993 strain produces both L-lysine and L-glutamic acid in the absence of any biotin action-suppressing agent even in the medium containing an excessive amount of biotin by shifting the cultivation temperature from 31.5.degree. C. to 34.degree. C., that the rate of L-glutamic acid increases in proportion to the earliness of the shift timing of the cultivation temperature, and that the rate of L-lysine increases in proportion to the lateness thereof. On the contrary, in the case of AJ11446 strain as the parent strain, only L-lysine was produced, and production of L-glutamic acid was not observed even by shifting the cultivation temperature.

Bacterial cells were removed by centrifugation from 1 l of cultivation-finished culture liquid having been subjected to the temperature shift 8 hours after the start of cultivation. L-lysine and L-glutamic acid were separated and purified from an obtained supernatant in accordance with an ordinary method using ion exchange resins. Crystals of obtained L-lysine hydrochlorid were 31.7 g, and crystals of obtained sodium L-glutamate were 13.9 g.

2. Investigation on Shift Temperature

Cultivation of Brevibacterium lactofermentum AJ11446 and AJ12993 was started at 31.5.degree. C. by using a jar fermenter made of glass having a volume of 500 ml in the same manner as the item 1. described above. The cultivation temperature was shifted to 33.degree. C., 34.degree. C. or 35.degree. C., 8 hours after the start of cultivation. A control for comparison was provided in which the cultivation temperature was not shifted to continue cultivation exactly at 31.5.degree. C. In any experiment, the cultivation was finished after 40–50 hours had passed. The amounts of L-lysine and L-glutamic acid produced and accumulated in the culture liquid were measured. Results are shown in Table 21.

TABLE 21

	AJ11446		AJ12993	
Temperature after	Lys	Glu	Lys	Glu

shift (.degree.C.)	(g/dl)	(g/dl)	(g/dl)	(g/dl)
33	5.6	0.0	4.9	1.1
34	5.4	0.0	4.4	1.8
35	5.2	0.0	3.8	2.1
—	5.9	0.0	6.0	0.0

According to the results, it is understood that there is a tendency that the rate of L-glutamic acid increases as the temperature after the shift becomes high, and the rate of L-lysine increases as it becomes low when AJ12993 strain is cultivated and the cultivation temperature is shifted.

INDUSTRIAL APPLICABILITY

to the invention, the temperature sensitivity to the biotin action-suppressing agent is given to the coryneform L-glutamic acid-producing bacteria. Thus L-glutamate can be produced inexpensively and stably by fermentation even when a material containing an excessive amount of biotin is used as a carbon source.

Further, the L-lysine productivity is given. Thus L-lysine and L-glutamate can be simultaneously produced inexpensively and stably by fermentation even when a material containing an excessiv amount of biotin is used as a carbon source.

SEQUENCE LISTING
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(iii) NUMBER OF SEQUENCES: 12
(2) INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2855 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Brevibacterium lactofermentum
(B) STRAIN: ATCC13869
(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 359..1987
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ValMetGlySerLysGlyLeuGlySerAspIleAsnLeuAlaTrpPro
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465470475480
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500505510
SerGluThrArgGlyGlnIleSerArgAsnLeuArgLeuLeuLysHis
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(ii) MOLECULE TYPE: other..syntetic DNA
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(iv) ANTI-SENSE: YES
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:
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D2

(54) Method of producing L-glutamic acid by fermentation

Verfahren zur Herstellung von Glutaminsäure durch Fermentation

Procédé de préparation de l'acide glutamique par fermentation

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(56) References cited:
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Description

Field of the Invention

[0001] The present invention relates to a mutant useful for producing L-glutamic acid by fermentation as well as a method of producing L-glutamic acid by fermentation using such a mutant. L-glutamic acid is an amino acid widely used as an additive for foods and in medicaments.

Prior Art

[0002] L-glutamic acid has conventionally been produced by fermentation using glutamic acid-producing bacteria and mutants thereof such as those of the genus Brevibacterium, Corynebacterium or Microbacterium (Amino acid fermentation, Gakkai Shuppan Center, pp.195 to 215 (1986)). Other known methods of producing L-glutamic acid by fermentation include a method employing microorganisms of the genus Bacillus, Streptomyces or Penicillium (US Patent No. 3,220,929) and a method employing microorganisms of the genus Pseudomonas, Arthrobacter, Serratia or Candida (US Patent No. 3,563,857). Even though such conventional methods produce significantly large amounts of L-glutamic acid, an even more efficient and less expensive method of producing L-glutamic acid is desired in order to meet the ever-increasing demand.

[0003] Escherichia coli is a potentially excellent L-glutamic acid-producing bacterium in view of its high growth rate and the availability of sufficient gene information, while the reported amount of L-glutamic acid production by Escherichia coli is as low as 2.3 g/l (J. Biochem., Vol. 50, pp.164 to 165 (1961)). Recently, a mutant exhibiting a deficient or reduced α-ketoglutarate dehydrogenase (hereinafter referred to as α-KGDH) was reported to have the ability to produce large amounts of L-glutamic acid (French Patent Application Laid-Open No. 2680178).

Problems to be Solved by the Invention

[0004] An objective of the present invention is to enhance the L-glutamic acid-producing ability of strains belonging to the genus Escherichia and to provide a method of producing L-glutamic acid more efficiently and at a lower cost.

Means to Solve the Problems

[0005] Now it has been found surprisingly in our study on the production of L-glutamic acid by mutants of Escherichia coli that a mutant whose α-KGDH activity is deficient or reduced, and whose phosphoenolpyruvate carboxylase (hereinafter referred to as PPC) and glutamate dehydrogenase (hereinafter referred to as GDH) activities are enhanced, has a high L-glutamic acid-producing ability, and thus the present invention has been accomplished.

[0006] Accordingly, the present invention relates to :

[0007] A mutant of the genus Escherichia having L-glutamic acid-producing ability whose α-KGDH activity is deficient or reduced, and PPC and GDH activities are enhanced; and,

[0008] A method of producing L-glutamic acid by fermentation comprising culturing in a liquid culture medium a mutant of the genus Escherichia having L-glutamic acid-producing ability whose α-KGDH activity is deficient or reduced and PPC and GDH activities are enhanced, accumulating L-glutamic acid in the culture, and recovering L-glutamic acid therefrom.

[0009] The present invention is described in more detail below.

(1) Derivation of a mutant of the genus Escherichia exhibiting deficient or reduced α-KGDH activity

[0010] As a starting parent strain to be used for preparing the present mutant, any non-pathogenic strain of the genus Escherichia may be employed. Examples of such strains are listed below.

Escherichia coli K-12 (ATCC 10798)
Escherichia coli W3110 (ATCC 27325)
Escherichia coli B (ATCC 11303)
Escherichia coli W (ATCC 9637)

[0011] A mutant of the genus Escherichia which has L-glutamic acid-producing ability and having deficient or reduced α-KGDH activity may be prepared as follows.

[0012] The starting parent strain mentioned above is first exposed to X-radiation or ultraviolet light or mutagenic agents such as N-methyl-N'-nitro-N-nitrosoguanidine (hereinafter referred to as NG) to introduce the mutation.

[0013] Alternatively, gene engineering technology, for example, gene recombination, gene transformation or cell fusion, may be used to efficiently introduce the intended mutation.

[0014] A method of obtaining an α-KGDH-deficient mutant by means of gene recombination is conducted as follows. Based on the known nucleotide sequence (Euro. J. Biochem. Vol. 141, pp. 351 to 359 (1984)) of α-ketoglutarate dehydrogenase gene (hereinafter referred to as sucA gene), primers are synthesized and then the sucA gene is amplified by the PCR method using the chromosomal DNA as a template. Into the amplified the sucA gene, a drug-resistant gene is inserted to obtain a sucA gene whose function is lost. Subsequently, using homologous recombination, the sucA gene on the chromosome is replaced by a sucA gene whose function is lost by means of the insertion of the drug-resistant gene.

[0015] After subjecting the parent strain to mutagenic treatment, the intended mutants may be screened by procedures as illustrated below.

[0016] A mutant exhibiting a deficient or reduced α-KGDH activity is either not able to grow or is able to grow only at a significantly reduced growth rate in a minimum culture medium containing glucose as the carbon source under aerobic condition. However, even under such condition, normal growth is possible by adding succinic acid or lysine plus methionine to the minimum culture medium containing glucose. On the other hand, anaerobic condition allows the mutant to grow even in the minimum culture medium containing glucose (Molec. Gen. Genetics, Vol. 105, pp. 182 to 190 (1969)). Based on these findings, the desired mutants can be screened.

[0017] The following strain is an example of the mutants thus obtained whose α-KGDH activity is deficient or reduced and which are listed below.

Escherichia coli W3110 sucA::Km^r

[0018] A mutant whose α-KGDH activity is deficient or reduced is more useful in view of its enhanced ability to produce L-glutamic acid when it further has the properties that L-glutamic acid-degrading activity is reduced or the expression of ace operon, that is, malate synthase (aceB) - isocitrate lyase (aceA) - isocitrate dehydrogenase kinase/phosphatase (aceK)-operon becomes constitutive. These properties are discussed in French Patent Application Laid-open No. 2680178. As a matter of course, properties already known to be effective for improving L-glutamic acid-productivity, such as various types of auxotrophy, antimetabolite resistance and antimetabolite sensitivity, are also desirable for enhancing L-glutamic acid production ability.

[0019] A mutant having reduced ability to degrade L-glutamic acid may be isolated as a mutant which either cannot grow or can grow only slightly in a minimum culture medium containing L-glutamic acid as the sole carbon source instead of glucose or containing L-glutamic acid as a sole nitrogen source instead of ammonium sulfate. However, as a matter of course, when an auxotroph is employed for the derivation, the minimum essential amount of the nutrient required for the growth may be added to the culture medium.

[0020] A mutant in which the expression of the ace operon is constitutive may be obtained as a strain whose parent strain is a phosphoenolpyruvate synthase-deficient strain and which can grow in a minimum culture medium containing lactic acid as the carbon source but cannot grow in a minimum culture medium containing pyruvic acid or acetic/pyruvic acid as the carbon source, or as a strain which shows a higher growth rate than that of its parent strain whose α-KGDH is deficient or reduced under aerobic condition (J. Bacteriol., Vol. 96, pp. 2185 to 2186 (1968)).

[0021] Examples of the mutants described above are as follows.

Escherichia coli AJ 12628 (FERM BP-3854)

Escherichia coli AJ 12624 (FERM BP-3853)

Escherichia coli AJ 12628 is a mutant having a reduced α-KGDH activity and a reduced ability to degrade L-glutamic acid in combination with constitutive expression of ace operon. Escherichia coli AJ 12624 is a mutant having reduced α-KGDH activity and a reduced ability to degrade L-glutamic acid (French Patent Application Laid-open No. 2680178).

[0022] In the mutant thus obtained which exhibits deficient or reduced α-KGDH activity, the flow of biosynthesis of L-glutamic acid via a -ketoglutaric acid in the TCA cycle is improved, resulting in an enhanced ability of producing L-glutamic acid. Also the productivity of L-glutamic acid is increased in the mutant exhibiting deficient or reduced α-KGDH activity and significantly low ability to degrade the produced L-glutamic acid or in the mutant further having a constitutive expression of the ace operon whereby the growth is improved.

(2) Derivation of a mutant of the genus Escherichia having amplified PPC activity and GDH activity

[0023] In the examples described below, a mutant of the genus Escherichia having amplified PPC and GDH activities was obtained from a starting parent strain exhibiting deficient or reduced α-KGDH activity and having the ability to produce L-glutamic acid. It is also possible to use a wild strain of the genus Escherichia as the parent strain to obtain a mutant having amplified PPC and GDH activities whereafter a mutant is bred which exhibits deficient or reduced α-KGDH activity.

[0024] Accordingly, the starting parent strain used to prepare a mutant having amplified PPC and GDH activities is preferably a mutant of the genus Escherichia whose α-KGDH activity is deficient or reduced and which has the ability to produce L-glutamic acid or a non-pathogenic wild type strain of the genus Escherichia. Examples of such strains

are listed below.

Escherichia coli W3100 sucA::Km^r
Escherichia coli AJ 12628 (FERM BP-3854)
Escherichia coli AJ 12624 (FERM BP-3853) (Those listed above are the mutants of the genus Escherichia whose α-KGDH activity is deficient or reduced and which have the ability to produce L-glutamic acid.)
Escherichia coli K-12 (ATCC 10798)
Escherichia coli W3110 (ATCC 27325)
Escherichia coli B (ATCC 11303)
Escherichia coli W (ATCC 9637)
(Those listed above are the non-pathogenic wild strains of the genus Escherichia.)

[0025] In order to amplify PPC and GDH activities, the genes coding for PPC and GDH are cloned in an appropriated plasmid, which is then used to transform the starting parent strain employed as a host. The copies of the genes coding for PPC and GDH (hereinafter referred to as ppc gene and gdhA gene, respectively) in the transformed cells are increased, resulting in amplified PPC and GDH activities.

[0026] The ppc gene and gdhA gene to be cloned may be cloned into a single plasmid to be introduced into the starting parent strain employed as the host, or may be cloned separately into two types of plasmid which are compatible in the starting parent strain.

[0027] Alternatively, the amplification of PPC and GDH activities may be conducted by allowing the ppc and gdhA genes to be present as multicopies on the chromosomal DNA of the starting parent strain employed as the host. In order to introduce the ppc and gdhA genes as multicopies into the chromosomal DNA of the genus Escherichia, homologous recombination is applied utilizing a target sequence present as a multicopy on the chromosomal DNA. The sequence present as the multicopy may be a repetitive DNA and an inverted repeat present at the terminal of insertion sequence. Alternatively, as described in Japanese Patent Application Laid-open No. 2-109985, the ppc and gdhA genes are cloned on a transposon, which is then transposed, thereby introducing the multicopy into the chromosomal DNA. The copies of the ppc and gdhA genes in the transformed cells are increased, resulting in the amplification of PPC and GDH activities.

[0028] In addition to the gene amplification described above, the amplification of PPC and GDH activities may also be conducted by replacing the promoters of the ppc and gdhA genes with those having higher potencies. For example, lac promoter, trp promoter, trc promoter, tac promoter, P_R promoter and P_L promoter of a lambda phage are known to be strong promoters. By enhancing the expression of the ppc gene and of the gdhA gene, the PPC and GDH activities are amplified.

[0029] The ppc and gdhA genes can be obtained by isolating the genes which are complementary with regard to auxotrophy of the mutants which are either PPC or GDH deficient. Alternatively, since the nucleotide sequences of these genes of Escherichia coli are known (J. Biochem., Vol. 95, pp. 909 to 916 (1984); Gene, Vol. 27, pp. 193 to 199 (1984)), the primers are synthesized based on the nucleotide sequences and then the genes are obtained by the PCR method using the chromosomal DNA as the template.

(3) Production of L-glutamic acid by fermentation using a mutant of the genus Escherichia capable of producing L-glutamic acid which exhibits deficient or reduced α-KGDH activity and has amplified PPC and GDH activities

[0030] For the purpose of producing L-glutamic acid by fermentation using a mutant of the genus Escherichia capable of producing L-glutamic acid which exhibits deficient or reduced α-KGDH activity and has amplified PPC and GDH activities, a standard culture medium containing carbon sources, nitrogen sources, inorganic salts and, if necessary, organic trace nutrients such as amino acids and vitamins and a standard culture method may be employed. The carbon sources and the nitrogen sources employed in the culture medium may be any of those catabolized by the mutant employed.

[0031] The carbon sources may be saccharides such as glucose, glycerol, fructose, sucrose, maltose, mannose, galactose, starch hydrolysate and molasses, and organic acids such as acetic acid and citric acid may also be employed independently or in combination with other carbon sources.

[0032] The nitrogen sources may be ammonia and ammonium salts such as ammonium sulfate, ammonium carbonate, ammonium chloride, ammonium phosphate, ammonium acetate as well as nitrates.

[0033] The organic trace nutrients may be amino acids, vitamins, fatty acids and nucleic acids as they are or as contained in peptone, casamino acid, yeast extract, soy protein hydrolysate and the like. In cases of using an auxotroph the nutrient required for its growth should be supplemented.

[0034] The inorganic salts may be phosphate, magnesium salts, calcium salts, iron salts, manganese salts and the like.

[0035] Cultivation is conducted at a fermentation temperature from 20 to 45°C at a pH controlled to be in a range of from 5 to 9 with aeration. When the pH is controlled during the cultivation, calcium carbonate or alkali such as ammonia

gas may be added for neutralization. After culturing for from 10 hours to 4 days, a significant amount of L-glutamic acid is accumulated in the culture medium.

[0036] L-glutamic acid in the culture medium after cultivation may be recovered by any of the known methods. For example, the cells are removed from the culture medium, which is then concentrated and precipitated or subjected to ion exchange chromatography to obtain L-glutamic acid.

Brief Description of the Drawings

[0037]

Fig. 1 shows the construction procedure of pBR-sucAB,
Fig. 2 shows a procedure for disrupting the sucA gene on the chromosomal DNA of Escherichia coli W3110, and
Fig. 3 shows the construction procedure of pGK.

Examples

[0038] The present invention is further described by the following examples.

Example 1

(1) Cloning of sucA gene and dihydrolipoamide succinyl transferase gene of Escherichia coli

[0039] The nucleotide sequences of sucA gene and dihydrolipoamide succinyl transferase gene (hereinafter referred to as sucB gene) of Escherichia coli K12 are known. The known nucleotide sequences of sucA gene and sucB gene are disclosed in Euro. J. Biochem., Vol. 141, pp. 351 to 374 (1984), and also shown here as Sequ ID No. 7 in the sequence listing. The nucleotide sequence from the 327th through the 3126th base residues corresponds to ORF (open reading frame) of the sucA gene, while that from the 3143rd through the 4357th base residues corresponds to ORF of the sucB gene. According to the nucleotide sequences reported, primers shown in Sequ ID No.1 to 4 were synthesized and sucA and sucB genes were amplified by PCR method employing the chromosomal DNA of Escherichia coli W3110 as a template.

[0040] The synthetic primers used to amplify the sucA gene had the nucleotide sequences shown in Sequ ID No.1 and 2, and Sequ ID No.1 corresponds to the sequence consisting of the 45th through the 65th base residues in the nucleotide sequence figure of the sucA gene described in Euro. J. Biochem., Vol. 141, p. 354 (1984). It also corresponds to the sequence consisting of the 45th through the 65th base residues of the nucleotide sequence shown as Sequ ID No. 7.

[0041] Sequ ID No. 2 corresponds to the sequence consisting of the 3173rd through the 3193rd base residues in the nucleotide sequence figure of the sucB gene shown in Euro. J. Biochem., Vol. 141, p. 364 (1984). It also corresponds to the sequence consisting of the 3173rd through the 3193rd base residues of the nucleotide sequence shown as Sequ ID No. 7.

[0042] The synthetic primers used to amplify the sucB gene had the nucleotide sequences shown in Sequ ID No.3 and 4, and Sequ ID No. 3 corresponds to the sequence consisting of the 2178th through 2198th base residues in the nucleotide sequence figure of the sucA gene shown in Euro. J. Biochem., Vol. 141, p. 354 (1984). It also corresponds to the sequence consisting of the 2179th through the 2198th base residues of the nucleotide sequence shown as Sequ No. 7.

[0043] Sequ ID No. 4 corresponds to the sequence consisting of the 4566th through the 4591st base residues in the nucleotide sequence figure of the sucB gene shown in Euro. J. Biochem., Vol. 141, p. 364 (1984). It also corresponds to the sequence consisting of the 4566th through the 4591st base residues of the nucleotide sequence shown as Sequ ID No. 7. The sucA gene and the sucB gene form an operon.

[0044] The chromosomal DNA of Escherichia coli W3110 was recovered by a standard method (Seibutsukogaku Jikkensho, Ed. by Nippon Seibutsu Kogaku Kai, pp. 97 to 98, Baifukan (1992)).

[0045] The PCR reaction was carried out under the standard conditions described on page 8 of PCR Technology (Ed. by Henry Erlich, Stockton Press (1989)).

[0046] Both ends of PCR products thus produced were converted into blunt ends using T4 DNA polymerase and cloned into a vector pBR322 at the EcoRV site. The plasmid obtained by cloning the sucA gene into pBR322 was designated as pBR-sucA, and that constructed with sucB was designated as pBR-sucB. The plasmids thus obtained were introduced into Escherichia coli JM109 and the plasmids were prepared. Then the restriction maps were constructed and compared with the restriction maps of the sucA and sucB genes reported, thereby confirming that the genes which had been cloned were the sucA and sucB genes.

[0047] As shown in Fig. 1, pBR-sucB was digested with KpnI and EcoRI to prepare a DNA fragment containing the sucB gene. pBR-sucA was digested with KpnI and EcoRI to prepare a large fragment. Both fragments were ligated using T4 DNA ligase to produce pBR-sucAB.

(2) Disruption of the sucA gene on chromosomal DNA of Escherichia coli W3110

[0048] Fig. 2 outlines the disruption of the sucA gene on the chromosomal DNA of Escherichia coli W3110.

[0049] pBR-sucAB was digested with KpnI and T4 DNA polymerase was used to obtain blunt ends. On the other hand, pUC4K (purchased from Pharmacia) was digested with PstI to prepare a DNA fragment containing a kanamycin-resistance gene, which was converted to have blunt ends using T4 DNA polymerase. Both fragments were ligated using T4 DNA ligase to obtain pBR-sucA::Km^r. From this plasmid, a HindIII-EcoRI fragment containing the kanamycin-resistance gene was cut out as a linear DNA, which was used to transform Escherichia coli JC7623 (thr-1, ara-14, leuB6, Δ(gpt-proA)62, lacY1, tsx-23, supE44, galK2, λ$^-$, rac$^-$, sbcB15, hisG4, rfbD1, recB21, recC22, rpsL31, kdgK51, xyl-5, mtl-1, argE3, thi-1) obtained from the Escherichia coli Genetic Stock Center (at Yale University, USA), and strains in which the sucA gene on the chromosomal DNA was replaced with the sucA gene into which the kanamycin-resistance gene had been inserted (sucA::Km^r) were screened on L medium (bactotrypton 10 g/l, yeast extract 5 g/l, NaCl 5 g/l, agar 15 g/l, pH 7.2) supplemented with 25 μg/ml of kanamycin. Since Escherichia coli JC7623 possessed recB$^-$, recC$^-$ and sbcB$^-$, recombination can be achieved at a high frequency even if the transformation is conducted using a linear DNA.

[0050] From each of twelve (12) kanamycin-resistant strains thus obtained, the chromosomal DNA was prepared and digested with KpnI. By southern hybridization using a DNA fragment containing the sucA gene as a probe, all of 12 strains were confirmed to be strains in which the sucA gene on the chromosomal DNA was replaced with the sucA gene into which kanamycin-resistance gene had been inserted. While a wild strain exhibits two bands at 5.2 Kb and 8.2 Kb due to the presence of KpnI site in the DNA fragment containing the sucA gene when a 2.8 Kb EcoRI-HindIII fragment containing the sucA gene of pBR-sucA was used as the probe in the southern hybridization, strains having the replacement with sucA gene into which kanamycin-resistance gene has been inserted exhibits only one band at 11.4 Kb due to the disruption of the KpnI site upon introduction of the kanamycin-resistance gene. The kanamycin-resistance Escherichia coli JC7623 (sucA::Km^r) thus obtained was then infected with P1 phage and the phage lysate was prepared. Then Escherichia coli W3100 strain was transduced with the sucA::Km^r. Transduction with P1 phage was conducted by a standard method (Seibutsu-kogaku Jikkensho, Ed. by Nippon Seibutsu Kogaku Kai, pp. 75 to 76, Baifukan (1992)). The representative of the kanamycin-resistance strains isolated was designated as W3110 sucA::Km^r.

[0051] The α-KGDH activities of the strain W3110 sucA::Km^r and Escherichia coli W3110 were determined according to the method by Reed et al (Methods in Enzymology, Vol. 13, pp. 55 (1969)). The resultant showed that the activity of Escherichia coli W3110 sucA::Km^r was not detected. Thus, Escherichia coli W3110 sucA::Km^r is a strain deficient in α-KGDH activity.

Table 1

	W3110	W3110sucA::Km^r
α-KGDH activity	3.70	Not detected
(Unit : micromoles/mg protein/min)		

(3) Cloning of gdhA gene of Escherichia coli W3110

[0052] Similarly as in the cloning of the sucA and sucB genes, the PCR method was used to clone the gdhA gene. According to the nucleotide sequence of gdhA gene reported by Fernando et al, primers for PCR were synthesized. The nucleotide sequence of the gdhA gene is disclosed in Gene, Vol. 27, pp.193 to 199 (1984), and is also shown here as Sequ ID No. 8 in the sequence listings. The nucleotide sequences of the primers are shown in Sequ ID Nos. 5 and 6.

[0053] Sequ ID No. 5 corresponds to the sequence from the 191st through the 171st base residues in the nucleotide sequence figure of gdhA gene shown in Gene, Vol. 27, p.195 (1984), and it also corresponds to the sequence from the 3rd through the 23rd base residues in Sequ ID No. 8.

[0054] Sequ ID No. 6 corresponds to the sequence consisting of the 1687th through the 1707th base residues in the nucleotide sequence figure of the gdhA gene shown in Gene, Vol. 27, p.195, (1984), and it also corresponds to the sequence consisting of 1880th through the 1900th base residues in Sequ ID No. 8.

[0055] Using the synthetic primers the gdhA gene was amplified by the PCR method employing the chromosomal DNA of Escherichia coli W3110 as a template. PCR products thus obtained were purified and converted to have blunt ends using T4 DNA polymerase, and then ligated to pBR 322 digested with EcoRV to obtain a plasmid pBRGDH.

(4) Construction of a plasmid having the ppc and gdhA genes

[0056] Fig. 3 shows the procedure for the construction of a plasmid having the ppc and gdhA genes. The plasmid pS2 in which 4.4Kb SalI fragment containing the whole region of the ppc gene derived from Escherichia coli K-12 was inserted into the SalI site of pBR322 (J. Biochem, Vol. 9, pp.909 to 916 (1984)) was digested with HindIII and both ends were made blunt using T4 DNA polymerase. On the other hand, a DNA fragment containing the gdhA gene synthesized by the PCR method was converted to have blunt ends using T4 DNA polymerase. Subsequently, both fragments were ligated using T4 DNA ligase. The plasmid thus obtained was used to transform a GDH deficient strain, Escherichia coli PA 340 (thr-1, thuA2, leuB6, lacY1, supE44, gal-6, λ^-, gdh-1, hisG1, rfbD1, galP63, Δ(gltB-F), rpsL19, malT1(lambdaR), xyl-7, mtl-2, argH1, thi-1) obtained from the Escherichia coli Genetic Stock Center (at Yale University, USA) and an ampicillin-resistant strain which had lost its glutamic acid requirement for growth was isolated. From this strain, a plasmid was prepared and the restriction map was constructed, whereby it was confirmed that the ppc and gdhA genes were present on this plasmid. This plasmid was designated as pGK.

(5) Introduction of pS2, pBRGDH and pGK into α-KGDH deficient strain Escherichia coli W3100 sucA::Km^r and evaluation of L-glutamic acid-production

[0057] The α-KGDH-deficient strain, Escherichia coli W3100 sucA::Km^r was transformed with each of pS2, pBRGDH and pGK, and each of the transformed strains was inoculated into a 500-ml shaker flask containing 20 ml of the culture medium having the composition shown in Table 2. Cultivation was then carried out at 37 °C until the glucose in the culture medium was consumed completely. The results are shown in Table 3.

Table 2

Component	Concentration (g/l)
Glucose	40
$(NH_4)_2SO_4$	20
KH_2PO_4	1
$MgSO_4 \cdot 7H_2O$	1
$FeSO_4 \cdot 7H_2O$	0.01
$MnSO_4 \cdot 5H_2O$	0.01
Yeast extract	2
Thiamine hydrochloride	0.01
$CaCO_3$	50

Table 3

Strain	Accumulated L-glutamic acid (g/l)
W3110 sucA::Km^r	19.2
W3110 sucA::Km^r/pS2	19.9
W3110 sucA::Km^r/pBRGDH	2.8
W3110 sucA::Km^r/pGK (AJ 12949)	23.3

[0058] Although the transformed strain having the PPC activity amplified by the introduction of pS2 exhibited slightly reduced growth as compared with the host strain, W3110 sucA::Km^r, it accumulated L-glutamic acid in an amount similar to that accumulated by the host strain. The strain having GDH activity amplified by the introduction of pBRGDH exhibited quite poor growth, and the amount of the accumulated L-glutamic acid was surprisingly smaller than that accumulated by the strain W3110 sucA::Km^r.

[0059] On the contrary, the transformed strain in which both of PPC and GDH activities were amplified simultaneously by the introduction of pGK exhibited growth similar to that of the host strain while producing an increased amount of accumulated L-glutamic acid. Escherichia coli W3110 sucA::Km^r into which pGK plasmid having the ppc and gdhA genes had been introduced was designated as AJ 12949. Escherichia coli AJ 12949 was originally deposited under the accession number FERM P-14039 on December 28, 1993, at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305, Japan, and the deposit was converted into a deposit under the Budapest Treaty under the accession number FERM BP-4881

on November 11, 1994.

[0060] The host strain, namely, W3110 sucA::Km^r can be obtained by curing the plasmid from the deposited strain, AJ 12949 without damaging the cell. The plasmid may be lost from AJ 12949 spontaneously, or may be cured in a curing procedure (Bact. Rev., Vol. 36, p.361 to 405 (1972)). An example of the curing procedure is as follows. The strain AJ 12949 is inoculated to the L-broth medium (Bactotrypton 10 g/l, yeast extract 5 g/l, NaCl 5 g/l, pH 7.2), and cultivated at 40°C overnight. The culture broth is diluted appropriately, and spread onto the L-medium. After incubating it at 37° C overnight, the colonies formed are transferred to the L-medium containing 100 µg/ml of ampicillin and then ampicillin-sensitive colonies are isolated. The strain thus obtained is W3110 sucA::Km^r.

Advantages of the Invention

[0061] The method according to the present invention provides a mutant of the genus Escherichia having a higher productivity of L-glutamic acid as well as the efficient and low-cost method for the production of L-glutamic acid.

SEQUENCE LISTING

GENERAL INFORMATION:

[0062] APPLICANT:
NAME: Ajinomoto Co., Inc.
STREET: 15-1, Kyobashi 1-chome
CITY: Chuo-ku, Tokyo
COUNTRY: Japan
POSTAL CODE: none

TITLE OF INVENTION: Method of producing L-glutamic acid by fermentation

NUMER OF SEQUENCES: 8

[0063] COMPUTER READABLE FORM:
MEDIUM TYPE: Diskette
COMPUTER: IBM PC compatible
OPERATING SYSTEM: MS-DOS

[0064] SEQUENCE DESCRIPTION:
SEQ ID No.: 1
Length : 21 base pairs
Type : Nucleotide
Strandedness : Single
Topology : Linear
Molecule type: Synthetic DNA
Feature : Primer for amplification of sucA gene of Escherichia coli

Sequence
ACGCGCAAGC GTCGCATCAG G 21

[0065] SEQ ID No.: 2
Length : 21 base pairs
Type : Nucleotide
Strandedness : Single
Topology : Linear
Molecule type: Synthetic DNA
Feature : Primer for amplification of sucA gene of Escherichia coli

Sequence
ATCGGCTACG AATTCAGGCA G 21

SEQ ID No.: 3
Length : 20 base pairs
Type : Nucleotide
Strandedness : Single
Topology : Linear
Molecule type: Synthetic DNA
Feature : Primer for amplification of sucB gene of Escherichia coli

Sequence
CCGGTCGCGG TACCTTCTTC 20

SEQ ID No.: 4
Length : 26 base pairs
Type : Nucleotide
Strandedness : Single
Topology : Linear
Molecule type: Synthetic DNA
Feature : Primer for amplification of sucB gene of Escherichia coli

Sequence
CGTAGACCGA ATTCTTCGTA TCGCTT 26

SQ ID No.: 5
Length : 21 base pairs
Type : Nucleotide
Strandedness : Single
Topology : Linear
Molecule type: Synthetic DNA
Feature : Primer for amplification of gdhA gene of Escherichia coli

Sequence
GGGTGGCAAA GCTTTAGCGT C 21

SEQ ID No.: 6
Length : 21 base pairs
Type : Nucleotide
Strandedness : Single
Topology : Linear
Molecule type: Synthetic DNA
Feature : Primer for amplification of gdhA gene of Escherichia coli

Sequence
TCGAGAAGCA TGCATTATAT A 21

SEQ ID No.: 7
Length : 4623 base pairs
Type : Nucleotide
Strandedness : Single
Topology : Linear
Molecule type: Genomic DNA
Original source
Organism : Escherichia coli
Features
Feature key : CDS ⇒from 327 to 318 bp coding sequence

Location : 327..3128
Method of feature determination : E
Feature key : CDS ⇒from 3143 to 4357 bp coding sequence Location : 3143..4357
Method of feature determination : E

Sequence

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TCGATGTTGT TGCAACGTAA TGCGTAAACC GTAGGCCTGA TAAGACGCGC AAGCGTCGCA      60
TCAGGCAACC AGTGCCGGAT GCGCGTGAAC GCCTTATCCG GCCTACAAGT CATTACCCGT     120
AGGCCTGATA AGCGCAGCGC ATCAGGCGTA ACAAAGAAAT GCAGGAAATC TTTAAAAACT     180
GCCCCTGACA GTAAGACAGT TTTTAAAGGT TCCTTCGCGA GCCACTACGT AGACAAGAGC     240
TCGCAAGTGA ACCCCGGCAC GCACATCACT GTGCGTGGTA GTATCCACGG CGAAGTAAGC     300
ATAAAAAAGA TGCTTAAGGG ATCACG ATG CAG AAC AGC GCT TTG AAA GCC TGG       353
                             Met Gln Asn Ser Ala Leu Lys Ala Trp
                              1               5

TTG GAC TCT TCT TAC CTC TCT GGC GCA AAC CAG AGC TGG ATA GAA CAG       401
Leu Asp Ser Ser Tyr Leu Ser Gly Ala Asn Gln Ser Trp Ile Glu Gln
 10              15                  20                  25
CTC TAT GAA GAC TTC TTA ACC GAT CCT GAC TCG GTT GAC GCT AAC TGG       449
Leu Tyr Glu Asp Phe Leu Thr Asp Pro Asp Ser Val Asp Ala Asn Trp
             30                  35                  40
CGT TCG ACG TTC CAG CAG TTA CCT GGT ACG GGA GTC AAA CCG GAT CAA       497
Arg Ser Thr Phe Gln Gln Leu Pro Gly Thr Gly Val Lys Pro Asp Gln
         45                  50                  55
TTC CAC TCT CAA ACG CGT GAA TAT TTC CGC CGC CTG GCG AAA GAC GCT       545
Phe His Ser Gln Thr Arg Glu Tyr Phe Arg Arg Leu Ala Lys Asp Ala
     60                  65                  70
TCA CGT TAC TCT TCA ACG ATC TCC GAC CCT GAC ACC AAT GTG AAG CAG       593
Ser Arg Tyr Ser Ser Thr Ile Ser Asp Pro Asp Thr Asn Val Lys Gln
 75                  80                  85
GTT AAA GTC CTG CAG CTC ATT AAC GCA TAC CGC TTC CGT GGT CAC CAG       641
Val Lys Val Leu Gln Leu Ile Asn Ala Tyr Arg Phe Arg Gly His Gln
 90              95                  100                 105
CAT GCG AAT CTC GAT CCG CTG GGA CTG TGG CAG CAA GAT AAA GTG GCC       689
His Ala Asn Leu Asp Pro Leu Gly Leu Trp Gln Gln Asp Lys Val Ala
            110                 115                 120
GAT CTG GAT CCG TCT TTC CAC GAT CTG ACC GAA GCA GAC TTC CAG GAG       737
Asp Leu Asp Pro Ser Phe His Asp Leu Thr Glu Ala Asp Phe Gln Glu
        125                 130                 135
ACC TTC AAC GTC GGT TCA TTT GCC AGC GGC AAA GAA ACC ATG AAA CTC       785
Thr Phe Asn Val Gly Ser Phe Ala Ser Gly Lys Glu Thr Met Lys Leu
    140                 145                 150
GGC GAG CTC CTG GAA GCC CTC AAG CAA ACC TAC TGC GGC CCG ATT GGT       833
Gly Glu Leu Leu Glu Ala Leu Lys Gln Thr Tyr Cys Gly Pro Ile Gly
155                 160                 165
GCC GAG TAT ATG CAC ATT ACC AGC ACC GAA GAA AAA CGC TGG ATC CAA       881
Ala Glu Tyr Met His Ile Thr Ser Thr Glu Glu Lys Arg Trp Ile Gln
170                 175                 180                 185
CAG CGT ATC GAG TCT GGT CGC GCG ACT TTC AAT AGC GAA GAG AAA AAA       929
Gln Arg Ile Glu Ser Gly Arg Ala Thr Phe Asn Ser Glu Glu Lys Lys
            190                 195                 200
CGC TTC TTA AGC GAA CTG ACC GCC GCT GAA GGT CTT GAA CGT TAC CTC       977
Arg Phe Leu Ser Glu Leu Thr Ala Ala Glu Gly Leu Glu Arg Tyr Leu
        205                 210                 215
GGC GCA AAA TTC CCT GGC GCA AAA CGC TTC TCG CTG GAA GGC GGT GAC      1025
Gly Ala Lys Phe Pro Gly Ala Lys Arg Phe Ser Leu Glu Gly Gly Asp
    220                 225                 230
GCG TTA ATC CCG ATG CTT AAA GAG ATG ATC CGC CAC GCT GGC AAC AGC      1073
Ala Leu Ile Pro Met Leu Lys Glu Met Ile Arg His Ala Gly Asn Ser
235                 240                 245
GGC ACC CGC GAA GTG GTT CTC GCG ATG GCG CAC CGT GGT CGT CTG AAC      1121
Gly Thr Arg Glu Val Val Leu Gly Met Ala His Arg Gly Arg Leu Asn
250                 255                 260                 265
GTG CTG GTG AAC GTG CTG GGT AAA AAA CCG CAA GAC TTG TTC GAC GAG      1169
Val Leu Val Asn Val Leu Gly Lys Lys Pro Gln Asp Leu Phe Asp Glu
            270                 275                 280
TTC GCC GGT AAA CAT AAA GAA CAC CTC GGC ACG GGT GAC GTG AAA TAC      1217
Phe Ala Gly Lys His Lys Glu His Leu Gly Thr Gly Asp Val Lys Tyr
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                285                 290                 295
CAC ATG GGC TTC TCG TCT GAC TTC CAG ACC GAT GGC GGC CTG GTG CAC   1265
His Met Gly Phe Ser Ser Asp Phe Gln Thr Asp Gly Gly Leu Val His
        300                 305                 310
CTG GCC CTG GCG TTT AAC CCG TCT CAC CTT GAG ATT GTA AGC CCG GTA   1313
Leu Ala Leu Ala Phe Asn Pro Ser His Leu Glu Ile Val Ser Pro Val
    315                 320                 325
GTT ATC GGT TCT GTT CGT GCC CGT CTG GAC AGA CTT GAT GAG CCG AGC   1361
Val Ile Gly Ser Val Arg Ala Arg Leu Asp Arg Leu Asp Glu Pro Ser
330                 335                 340                 345
AGC AAC AAA GTG CTG CCA ATC ACC ATC CAC GGT GAC GCC GCA GTG ACC   1409
Ser Asn Lys Val Leu Pro Ile Thr Ile His Gly Asp Ala Ala Val Thr
                350                 355                 360
GGG CAG GGC GTG GTT CAG GAA ACC CTG AAC ATG TCG AAA GCG CGT GGT   1457
Gly Gln Gly Val Val Gln Glu Thr Leu Asn Met Ser Lys Ala Arg Gly
            365                 370                 375
TAT GAA GTT GGC GGT ACG GTA CGT ATC GTT ATC AAC AAC CAG GTT GGT   1505
Tyr Glu Val Gly Gly Thr Val Arg Ile Val Ile Asn Asn Gln Val Gly
        380                 385                 390
TTC ACC ACC TCT AAT CCG CTG GAT GCC CGT TCT ACG CCG TAC TGT ACT   1553
Phe Thr Thr Ser Asn Pro Leu Asp Ala Arg Ser Thr Pro Tyr Cys Thr
    395                 400                 405
GAT ATC GGT AAG ATG GTT CAG GCC CCG ATT TTC CAC GTT AAC GCG GAC   1601
Asp Ile Gly Lys Met Val Gln Ala Pro Ile Phe His Val Asn Ala Asp
410                 415                 420                 425
GAT CCG GAA GCC GTT GCC TTT GTG ACC CGT CTG GCG CTC GAT TTC CGT   1649
Asp Pro Glu Ala Val Ala Phe Val Thr Arg Leu Ala Leu Asp Phe Arg
                430                 435                 440
AAC ACC TTT AAA CGT GAT GTC TTC ATC GAC CTG GTG TCG TAC CGC CGT   1697
Asn Thr Phe Lys Arg Asp Val Phe Ile Asp Leu Val Ser Tyr Arg Arg
            445                 450                 455
CAC GGC CAC AAC GAA GCC GAC GAG CCG AGC GCA ACC CAG CCG CTG ATG   1745
His Gly His Asn Glu Ala Asp Glu Pro Ser Ala Thr Gln Pro Leu Met
        460                 465                 470
TAT CAG AAA ATC AAA AAA CAT CCG ACA CCG CGC AAA ATC TAC GCT GAC   1793
Tyr Gln Lys Ile Lys Lys His Pro Thr Pro Arg Lys Ile Tyr Ala Asp
    475                 480                 485
AAG CTG GAG CAG GAA AAA GTG GCG ACG CTG GAA GAT GCC ACC GAG ATG   1841
Lys Leu Glu Gln Glu Lys Val Ala Thr Leu Glu Asp Ala Thr Glu Met
490                 495                 500                 505
GTT AAC CTG TAC CGC GAT GCG CTG GAT GCT GGC GAT TGC GTA GTG GCA   1889
Val Asn Leu Tyr Arg Asp Ala Leu Asp Ala Gly Asp Cys Val Val Ala
                510                 515                 520
GAG TGG CGT CCG ATG AAC ATG CAC TCT TTC ACC TGG TCG CCG TAC CTC   1937
Glu Trp Arg Pro Met Asn Met His Ser Phe Thr Trp Ser Pro Tyr Leu
            525                 530                 535
AAC CAC GAA TGG GAC GAA GAG TAC CCG AAC AAA GTT GAG ATG AAG CGC   1985
Asn His Glu Trp Asp Glu Glu Tyr Pro Asn Lys Val Glu Met Lys Arg
        540                 545                 550
CTG CAG GAG CTG GCG AAA CGC ATC AGC ACG GTG CCG GAA GCA GTT GAA   2033
Leu Gln Glu Leu Ala Lys Arg Ile Ser Thr Val Pro Glu Ala Val Glu
    555                 560                 565
ATG CAG TCT CGC GTT GCC AAG ATT TAT GGC GAT CGC CAG GCG ATG GCT   2081
Met Gln Ser Arg Val Ala Lys Ile Tyr Gly Asp Arg Gln Ala Met Ala
570                 575                 580                 585
GCC GGT GAG AAA CTG TTC GAC TGG GGC GGT GCG GAA AAC CTC GCT TAC   2129
Ala Gly Glu Lys Leu Phe Asp Trp Gly Gly Ala Glu Asn Leu Ala Tyr
                590                 595                 600
GCC ACG CTG GTT GAT GAA GGC ATT CCG GTT CGC CTG TCG GGT GAA GAC   2177
Ala Thr Leu Val Asp Glu Gly Ile Pro Val Arg Leu Ser Gly Glu Asp
            605                 610                 615
TCC GGT CGC GGT ACC TTC TTC CAC CGC CAC GCG GTG ATC CAC AAC CAG   2225
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S r Gly Arg Gly Thr Phe Phe His Arg His Ala Val Ile His Asn Gln
          620                 625                 630
TCT AAC GGT TCC ACT TAC ACG CCG CTG CAA CAT ATC CAT AAC GGG CAG      2273
Ser Asn Gly Ser Thr Tyr Thr Pro Leu Gln His Ile His Asn Gly Gln
    635                 640                 645
GGC GCG TTC CGT GTC TGG GAC TCC GTA CTG TCT GAA GAA GCA GTG CTG      2321
Gly Ala Phe Arg Val Trp Asp Ser Val Leu Ser Glu Glu Ala Val Leu
650                 655                 660                 665
GCG TTT GAA TAT GGT TAT GCC ACC GCA GAA CCA CGC ACT CTG ACC ATC      2369
Ala Phe Glu Tyr Gly Tyr Ala Thr Ala Glu Pro Arg Thr Leu Thr Ile
                670                 675                 680
TGG GAA GCG CAG TTC GGT GAC TTC GCC AAC GGT GCG CAG GTG GTT ATC      2417
Trp Glu Ala Gln Phe Gly Asp Phe Ala Asn Gly Ala Gln Val Val Ile
            685                 690                 695
GAC CAG TTC ATC TCC TCT GGC GAA CAG AAA TGG GGC CGG ATG TGT GGT      2465
Asp Gln Phe Ile Ser Ser Gly Glu Gln Lys Trp Gly Arg Met Cys Gly
        700                 705                 710
CTG GTG ATG TTG CTG CCG CAC GGT TAC GAA GGG CAG GGG CCG GAG CAC      2513
Leu Val Met Leu Leu Pro His Gly Tyr Glu Gly Gln Gly Pro Glu His
    715                 720                 725
TCC TCC GCG CGT CTG GAA CGT TAT CTG CAA CTT TGT GCT GAG CAA AAC      2561
Ser Ser Ala Arg Leu Glu Arg Tyr Leu Gln Leu Cys Ala Glu Gln Asn
730                 735                 740                 745
ATG CAG GTT TGT GTA CCG TCT ACC CCG GCA CAA GTT TAC CAC ATG CTC      2609
Met Gln Val Cys Val Pro Ser Thr Pro Ala Gln Val Tyr His Met Leu
                750                 755                 760
CGT CGT CAG GCG CTG CGC GGG ATG CGT CGT CCG CTG GTC GTG ATG TCG      2657
Arg Arg Gln Ala Leu Arg Gly Met Arg Arg Pro Leu Val Val Met Ser
            765                 770                 775
CCG AAA TCC CTG CTG CGT CAT CCG CTG GCG GTT TCC AGC CTC GAA GAA      2705
Pro Lys Ser Leu Leu Arg His Pro Leu Ala Val Ser Ser Leu Glu Glu
        780                 785                 790
CTG GCG AAC GGC ACC TTC CTG CCA GCC ATC GGT GAA ATC GAC GAG CTT      2753
Leu Ala Asn Gly Thr Phe Leu Pro Ala Ile Gly Glu Ile Asp Glu Leu
    795                 800                 805
GAT CCG AAG GGC GTG AAG CGC GTA GTG ATG TGT TCT GGT AAG GTT TAT      2801
Asp Pro Lys Gly Val Lys Arg Val Val Met Cys Ser Gly Lys Val Tyr
810                 815                 820                 825
TAC GAC CTG CTG GAA CAG CGT CGT AAG AAC AAT CAA CAC GAT GTC GCC      2849
Tyr Asp Leu Leu Glu Gln Arg Arg Lys Asn Asn Gln His Asp Val Ala
                830                 835                 840
ATT GTG CGT ATC GAG CAA CTC TAC CCG TTC CCG CAT AAA GCG ATG CAG      2897
Ile Val Arg Ile Glu Gln Leu Tyr Pro Phe Pro His Lys Ala Met Gln
            845                 850                 855
GAA GTG TTG CAG CAG TTT GCT CAC GTC AAG GAT TTT GTC TGG TGC CAG      2945
Glu Val Leu Gln Gln Phe Ala His Val Lys Asp Phe Val Trp Cys Gln
        860                 865                 870
GAA GAG CCG CTC AAC CAG GGC GCA TGG TAC TGC AGC CAG CAT CAT TTC      2993
Glu Glu Pro Leu Asn Gln Gly Ala Trp Tyr Cys Ser Gln His His Phe
    875                 880                 885
CGT GAA GTG ATT CCG TTT GGG GCT TCT CTG CGT TAT GCA GGC CGC CCG      3041
Arg Glu Val Ile Pro Phe Gly Ala Ser Leu Arg Tyr Ala Gly Arg Pro
890                 895                 900                 905
GCC TCC GCC TCT CCG GCG GTA GGG TAT ATG TCC GTT CAC CAG AAA CAG      3089
Ala Ser Ala Ser Pro Ala Val Gly Tyr Met Ser Val His Gln Lys Gln
                910                 915                 920
CAA CAA GAT CTG GTT AAT GAC GCG CTG AAC GTC GAA TAAATAAAGG           3135
Gln Gln Asp Leu Val Asn Asp Ala Leu Asn Val Glu
            925                 930
ATACACA ATG AGT AGC GTA GAT ATT CTG GTC CCT GAC CTG CCT GAA TCC      3184
        Met Ser Ser Val Asp Ile Leu Val Pro Asp Leu Pro Glu Ser
          1                   5                  10
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GTA GCC GAT GCC ACC GTC GCA ACC TGG CAT AAA AAA CCC GGC GAC GCA      3232
Val Ala Asp Ala Thr Val Ala Thr Trp His Lys Lys Pro Gly Asp Ala
 15                  20                  25                  30
GTC GTA CGT GAT GAA GTG CTG GTA GAA ATC GAA ACT GAC AAA GTG GTA      3280
Val Val Arg Asp Glu Val Leu Val Glu Ile Glu Thr Asp Lys Val Val
             35                  40                  45
CTG GAA GTA CCG GCA TCA GCA GAC GGC ATT CTG GAT GCG GTT CTG GAA      3328
Leu Glu Val Pro Ala Ser Ala Asp Gly Ile Leu Asp Ala Val Leu Glu
         50                  55                  60
GAT GAA GGT ACA ACG GTA ACG TCT CGT CAG ATC CTT GGT CGC CTG CGT      3376
Asp Glu Gly Thr Thr Val Thr Ser Arg Gln Ile Leu Gly Arg Leu Arg
 65                  70                  75
GAA GGC AAC AGC GCC GGT AAA GAA ACC AGC GCC AAA TCT GAA GAG AAA      3424
Glu Gly Asn Ser Ala Gly Lys Glu Thr Ser Ala Lys Ser Glu Glu Lys
 80                  85                  90
GCG TCC ACT CCG GCG CAA CGC CAG CAG GCG TCT CTG GAA GAG CAA AAC      3472
Ala Ser Thr Pro Ala Gln Arg Gln Gln Ala Ser Leu Glu Glu Gln Asn
 95                 100                 105                 110
AAC GAT GCG TTA AGC CCG GCG ATC CGT CGC CTG CTG GCT GAA CAC AAT      3520
Asn Asp Ala Leu Ser Pro Ala Ile Arg Arg Leu Leu Ala Glu His Asn
            115                 120                 125
CTC GAC GCC AGC GCC ATT AAA GGC ACC GGT GTG GGT GGT CGT CTG ACT      3568
Leu Asp Ala Ser Ala Ile Lys Gly Thr Gly Val Gly Gly Arg Leu Thr
        130                 135                 140
CGT GAA GAT GTG GAA AAA CAT CTG GCG AAA GCC CCG GCG AAA GAG TCT      3616
Arg Glu Asp Val Glu Lys His Leu Ala Lys Ala Pro Ala Lys Glu Ser
    145                 150                 155
GCT CCG GCA GCG GCT GCT CCG GCG GCG CAA CCG GCT CTG GCT GCA CGT      3664
Ala Pro Ala Ala Ala Ala Pro Ala Ala Gln Pro Ala Leu Ala Ala Arg
160                 165                 170
AGT GAA AAA CGT GTC CCG ATG ACT CGC CTG CGT AAG CGT GTG GCA GAG      3712
Ser Glu Lys Arg Val Pro Met Thr Arg Leu Arg Lys Arg Val Ala Glu
175                 180                 185                 190
CGT CTG CTG GAA GCG AAA AAC TCC ACC GCC ATG CTG ACC ACG TTC AAC      3760
Arg Leu Leu Glu Ala Lys Asn Ser Thr Ala Met Leu Thr Thr Phe Asn
            195                 200                 205
GAA GTC AAC ATG AAG CCG ATT ATG GAT CTG CGT AAG CAG TAC GGT GAA      3808
Glu Val Asn Met Lys Pro Ile Met Asp Leu Arg Lys Gln Tyr Gly Glu
        210                 215                 220
GCG TTT GAA AAA CGC CAC GGC ATC CGT CTG GGC TTT ATG TCC TTC TAC      3856
Ala Phe Glu Lys Arg His Gly Ile Arg Leu Gly Phe Met Ser Phe Tyr
    225                 230                 235
GTG AAA GCG GTG GTT GAA GCC CTG AAA CGT TAC CCG GAA GTG AAC GCT      3904
Val Lys Ala Val Val Glu Ala Leu Lys Arg Tyr Pro Glu Val Asn Ala
240                 245                 250
TCT ATC GAC GGC GAT GAC GTG GTT TAC CAC AAC TAT TTC GAC GTC AGC      3952
Ser Ile Asp Gly Asp Asp Val Val Tyr His Asn Tyr Phe Asp Val Ser
255                 260                 265                 270
ATG GCG GTT TCT ACG CCG CGC GGC CTG GTG ACG CCG GTT CTG CGT GAT      4000
Met Ala Val Ser Thr Pro Arg Gly Leu Val Thr Pro Val Leu Arg Asp
            275                 280                 285
GTC GAT ACC CTC GGC ATG GCA GAC ATC GAG AAG AAA ATC AAA GAG CTG      4048
Val Asp Thr Leu Gly Met Ala Asp Ile Glu Lys Lys Ile Lys Glu Leu
        290                 295                 300
GCA GTC AAA GGC CGT GAC GGC AAG CTG ACC GTT GAA GAT CTG ACC GGT      4096
Ala Val Lys Gly Arg Asp Gly Lys Leu Thr Val Glu Asp Leu Thr Gly
    305                 310                 315
GGT AAC TTC ACC ATC ACC AAC GGT GGT GTG TTC GGT TCC CTG ATG TCT      4144
Gly Asn Phe Thr Ile Thr Asn Gly Gly Val Phe Gly Ser Leu Met Ser
320                 325                 330
ACG CCG ATC ATC AAC CCG CCG CAG AGC GCA ATT CTG GGT ATG CAC GCT      4192
Thr Pro Ile Ile Asn Pro Pro Gln Ser Ala Ile Leu Gly Met His Ala
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335                 340                 345                 350
ATC AAA GAT CGT CCG ATG GCG GTG AAT GGT CAG GTT GAG ATC CTG CCG      4240
Ile Lys Asp Arg Pro Met Ala Val Asn Gly Gln Val Glu Ile Leu Pro
                355                 360                 365
ATG ATG TAC CTG GCG CTG TCC TAC GAT CAC CGT CTG ATC GAT GGT CGC      4288
Met Met Tyr Leu Ala Leu Ser Tyr Asp His Arg Leu Ile Asp Gly Arg
            370                 375                 380
GAA TCC GTG GGC TTC CTG GTA ACG ATC AAA GAG TTG CTG GAA GAT CCG      4336
Glu Ser Val Gly Phe Leu Val Thr Ile Lys Glu Leu Leu Glu Asp Pro
        385                 390                 395
ACG CGT CTG CTG CTG GAC GTG TAGTAGTTTA AGTTTCACCT GCACTGTAGA         4387
Thr Arg Leu Leu Leu Asp Val
    400                 405
CCGGATAAGG CATTATCGCC TTCTCCGGCA ATTGAAGCCT GATGCGACGC TGACGCGTCT    4447
TATCAGGCCT ACGGGACCAC CAATGTAGGT CGGATAAGGC GCAACGCCGC ATCCGACAAG    4507
CGATGCCTGA TGTGACGTTT AACGTGTCTT ATCAGGCCTA CGGGTGACCG ACAATGCCCG    4567
GAAGCGATAC GAAATATTCC GTCTACCGTT TAAAAGATAA CGATTACTGA AGGATG·····   4625
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SEQ ID No.: 8
Length : 1937 base pairs
Type : Nucleotide
Strandedness : Single
Topology : Linear
Molecule type: Genomic DNA
Original source
Organism : Escherichia coli
Sequence feature
Feature key : CDS ⇒from 194 to 1537 bp coding sequence Location : 194..1537
Method of feature determination : E

Sequence

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CCGGGTGGCA AAACTTTAGC GTCTGAGGTT ATCGCAATTT GGTTATGAGA TTACTCTCGT      60
TATTAATTTG CTTTCCTGGG TCATTTTTTT CTTGCTTACC GTCACATTCT TGATGGTATA     120
GTCGAAAACT GCAAAAGCAC ATGACATAAA CAACATAAGC ACAATCGTAT TAATATATAA     180
GGGTTTTATA TCT ATG GAT CAG ACA TAT TCT CTG GAG TCA TTC CTC AAC         229
              Met Asp Gln Thr Tyr Ser Leu Glu Ser Phe Leu Asn
                1               5                  10
CAT GTC CAA AAG CGC GAC CCG AAT CAA ACC GAG TTC GCC CAA GCC GTT       277
His Val Gln Lys Arg Asp Pro Asn Gln Thr Glu Phe Ala Gln Ala Val
            15                  20                  25
CGT GAA GTA ATG ACC ACA CTC TGG CCT TTT CTT GAA CAA AAT CCA AAA       325
Arg Glu Val Met Thr Thr Leu Trp Pro Phe Leu Glu Gln Asn Pro Lys
        30                  35                  40
TAT CGC CAG ATG TCA TTA CTG GAG CGT CTG GTT GAA CCG GAG CGC GTG       373
Tyr Arg Gln Met Ser Leu Leu Glu Arg Leu Val Glu Pro Glu Arg Val
    45                  50                  55                  60
ATC CAG TTT CGC GTG GTA TGG GTT GAT GAT CGC AAC CAG ATA CAG GTC       421
Ile Gln Phe Arg Val Val Trp Val Asp Asp Arg Asn Gln Ile Gln Val
                65                  70                  75
AAC CGT GCA TGG CGT GTG CAG TTC AGC TCT GCC ATC GGC CCG TAC AAA       469
Asn Arg Ala Trp Arg Val Gln Phe Ser Ser Ala Ile Gly Pro Tyr Lys
            80                  85                  90
GGC GGT ATG CGC TTC CAT CCG TCA GTT AAC CTT TCC ATT CTC AAA TTC       517
Gly Gly Met Arg Phe His Pro Ser Val Asn Leu Ser Ile Leu Lys Phe
        95                  100                 105
CTC GGC TTT GAA CAA ACC TTC AAA AAT GCC CTG ACT ACT CTG CCG ATG       565
Leu Gly Phe Glu Gln Thr Phe Lys Asn Ala Leu Thr Thr Leu Pro Met
    110                 115                 120
GGC GGT GGT AAA GGC GGC AGC GAT TTC GAT CCG AAA GGA AAA AGC GAA       613
Gly Gly Gly Lys Gly Gly Ser Asp Phe Asp Pro Lys Gly Lys Ser Glu
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125                 130                 135                 140
GGT GAA GTG ATG CGT TTT TGC CAG GCG CTG ATG ACT GAA CTG TAT CGC      661
Gly Glu Val Met Arg Phe Cys Gln Ala Leu Met Thr Glu Leu Tyr Arg
                145                 150                 155
CAC CTG GGC GCG GAT ACC GAC GTT CCG GCA GGT GAT ATC GGG GTT GGT      709
His Leu Gly Ala Asp Thr Asp Val Pro Ala Gly Asp Ile Gly Val Gly
            160                 165                 170
GGT CGT GAA GTC GGC TTT ATG GCG GGG ATG ATG AAA AAG CTC TCC AAC      757
Gly Arg Glu Val Gly Phe Met Ala Gly Met Met Lys Lys Leu Ser Asn
        175                 180                 185
AAT ACC GCC TGC GTC TTC ACC GGT AAG GGC CTT TCA TTT GGC GGC AGT      805
Asn Thr Ala Cys Val Phe Thr Gly Lys Gly Leu Ser Phe Gly Gly Ser
    190                 195                 200
CTT ATT CGC CCG GAA GCT ACC GGC TAC GGT CTG GTT TAT TTC ACA GAA      853
Leu Ile Arg Pro Glu Ala Thr Gly Tyr Gly Leu Val Tyr Phe Thr Glu
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GCA ATG CTA AAA CGC CAC GGT ATG GGT TTT GAA GGG ATG CGC GTT TCC      901
Ala Met Leu Lys Arg His Gly Met Gly Phe Glu Gly Met Arg Val Ser
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GTT TCT GGC TCC GGC AAC GTC GCC CAG TAC GCT ATC GAA AAA GCG ATG      949
Val Ser Gly Ser Gly Asn Val Ala Gln Tyr Ala Ile Glu Lys Ala Met
            240                 245                 250
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        255                 260                 265
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His Gln Leu Ile Ala Asn Gly Val Lys Ala Val Ala Glu Gly Ala Asn
        335                 340                 345
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                385                 390                 395
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Gly Val Ile
445
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TAAAGAAATC CCATTTGACT ATTTTTTTGA TAATCTTCTT CGCTTTCGAA CAACTCGTGC   1874
GCCTTTCGAG AAGCAAGCAT TATATAATGC CAGGCCAGTT CTTCTTCAAT TGTCCCGTTT   1934
TGA                                                                1937
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Claims

1. A mutant of the genus Escherichia having L-glutamic acid-productivity, said mutant having deficient or reduced α-ketoglutarate dehydrogenase activity and enhanced phosphoenolpyruvate carboxylase and glutamate dehydrogenase activities.

2. A method of producing L-glutamic acid by fermentation comprising culturing in a liquid culture medium a mutant of the genus Escherichia having L-glutamic acid-productivity said mutant having deficient or reduced α-ketoglutarate dehydrogenase activity and enhanced phosphoenolpyruvate carboxylase and glutamate dehydrogenase activities, accumulating L-glutamic acid in the culture, and recovering L-glutamic acid therefrom.

Patentansprüche

1. Mutante der Gattung Escherichia, die L-Glutaminsäure produziert und keine oder verminderte α-Ketoglutaratdehydrogenaseaktivität und erhöhte Phosphoenolpyruvatcarboxylase- und Glutamatdehydrogenaseaktivitäten hat.

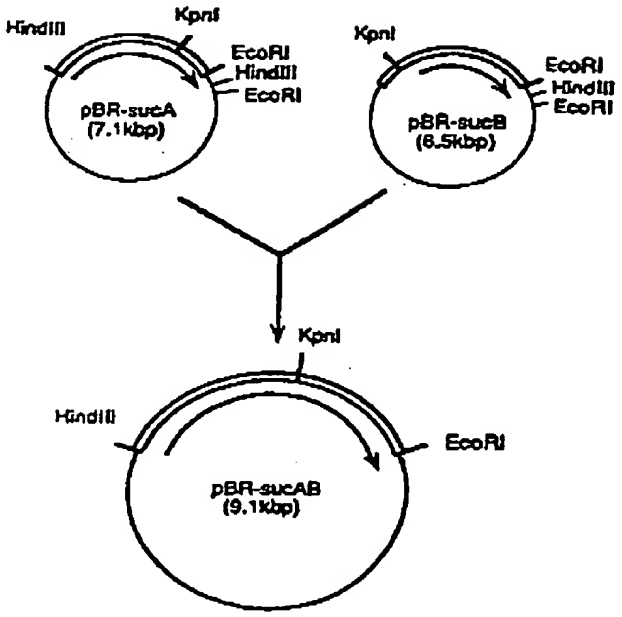
2. Verfahren zur Herstellung von L-Glutaminsäure durch Fermentation, welches das Kultivieren einer Mutante der Gattung Escherichia, die L-Glutaminsäure produziert und keine oder verminderte α-Ketoglutaratdehydrogenaseaktivität und erhöhte Phosphoenolpyruvatcarboxylase- und Glutamatdehydrogenaseaktivitäten hat, in einem flüssigen Kulturmedium, das Anhäufen von L-Glutaminsäure in der Kultur und das Gewinnen von L-Glutaminsäure daraus umfaßt.

Revendications

1. Mutant du genre Escherichia ayant une productivité d'acide L-glutamique, ledit mutant ayant une activité α-cétoglutarate déshydrogénase déficiente ou réduite et des activités phosphoénolpyruvate carboxylase et glutamate déshydrogénase augmentées.

2. Procédé de production d'acide L-glutamique par fermentation comprenant la culture dans un milieu de culture liquide d'un mutant du genre Escherichia ayant une productivité d'acide L-glutamique, ledit mutant ayant une activité α-cétoglutarate déshydrogénase déficiente ou réduite et des activités phosphoénolpyruvate carboxylase et glutamate déshydrogénase augmentées, l'accumulation d'acide L-glutamique dans la culture et la récupération de l'acide L-glutamique à partir de celle-ci.

Fig.1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KpnI
EcoRI
HindIII
EcoRI
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(7.1kbp)
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EcoRI
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(6.5kbp)
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HindIII
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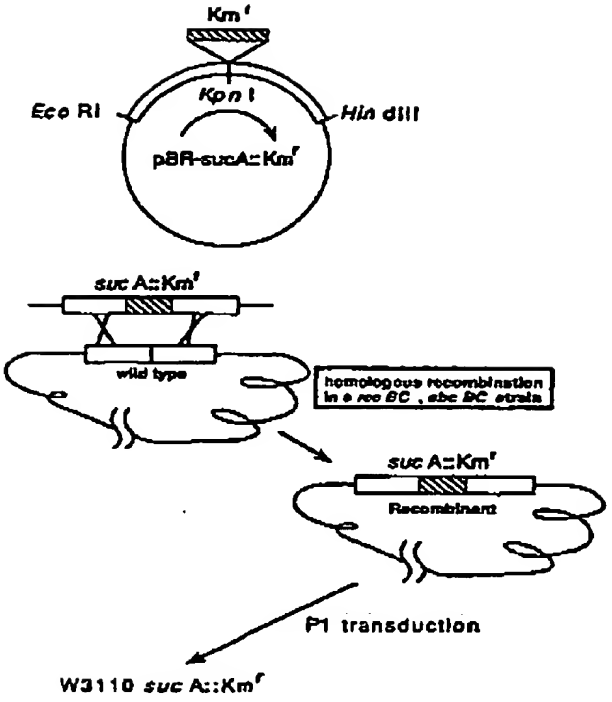
Fig. 2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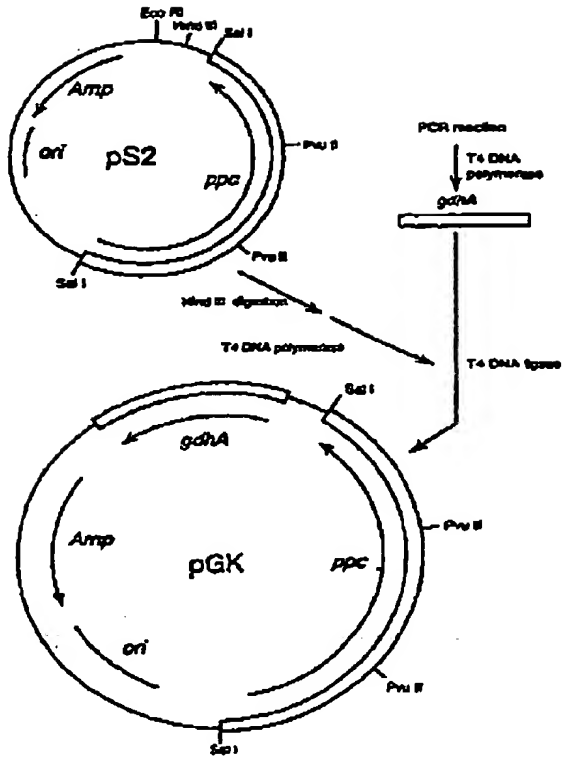
suc A::Km'
Recombinant
P1 transduction
W3110 suc A::Km'

Fig.3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Sal I
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gdhA
T4 DNA polymerase
T4 DNA ligase
gdhA
Amp
pGK
ppc
ori
Sal I

D3

ESCHERICHIA COLI AND SALMONELLA TYPHIMURIUM

CELLULAR AND MOLECULAR BIOLOGY

VOLUME 1

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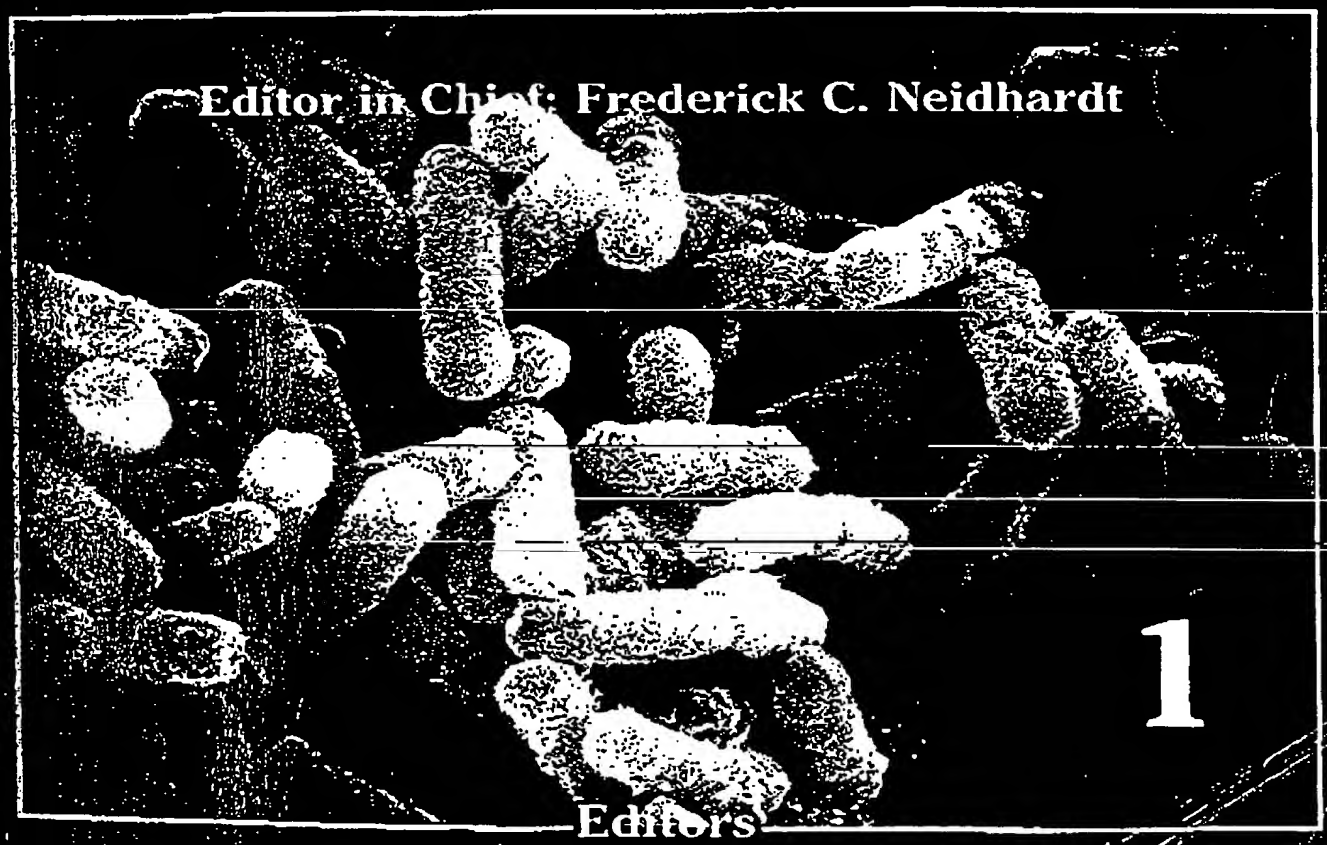

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ESCHERICHIA COLI AND SALMONELLA TYPHIMURIUM

CELLULAR AND MOLECULAR BIOLOGY

VOLUME 1

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14. Th Tricarboxylic Acid Cycle and Anaplerotic Reactions

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INTRODUCTION

The central metabolic pathways are those into which flow all carbon compounds used by the cell and from which all new cell material and waste products are derived. The discovery of the tricarboxylic acid (TCA) cycle in 1937 (57) and its subsequent acceptance as the terminal pathway for the oxidation of foodstuffs in all respiring animal tissues was one of the most significant events in the development of modern biochemistry. Attempts to demonstrate the operation of the TCA cycle in microorganisms were initially unsuccessful owing to various technical difficulties. These problems, and the evidence that finally established the cycle as the major pathway of terminal respiration in microorganisms, have been covered in earlier reviews (53, 58).

The reactions of the TCA cycle and its operation under aerobic conditions are summarized in Fig. 1. The cycle affords the oxidation of acetyl units to CO_2 and the generation of reduced nucleotides that are used for reductive biosynthesis or for trapping energy in the form of ATP. The cycle also provides many of the precursors required for biosynthesis (Fig. 1). This function, of course, dictates the requirement for the so-called anaplerotic reactions, the role of which is to replenish the intermediates of the cycle (see below). The nature of the carbon source dictates which anaplerotic reaction or pathway is used.

In bacteria under anaerobic conditions, the TCA cycle does not operate as shown in Fig. 1, but rather as two separate limbs emanating from oxaloacetate that generate the 2-oxoglutarate and succinyl-coenzyme A (CoA) required for biosynthesis (1). This branched, noncyclic pathway also seems to operate during aerobic growth on glucose, when the energy requirements of the cell are largely satisfied by glycolysis. Amarasingham and Davis (1) proposed that succinate formation from oxaloacetate involved reversal of flux through the TCA cycle enzymes malate dehydrogenase and fumarase, while the reduction of fumarate was catalyzed by a fumarate reductase (46), specifically induced by anaerobiosis, rather than by succinate dehydrogenase. Courtright and Henning (17) subsequently showed that malate dehydrogenase is not necessary for the anaerobic generation of succinate from oxaloacetate and suggested that fumarate formation from oxaloacetate occurs via aspartate. However, there is no evidence that this is the only pathway that leads to anaerobic generation of fumarate. Both of the possibilities described above are shown in Fig. 2.

The basic outline of the TCA cycle and its metabolic roles has thus been clear for many years. In this review, I concentrate on more recent findings in two main areas: first, the organization and expression of the genes encoding TCA cycle enzymes; second, the short-term control of the cycle and anaplerotic reactions.

MOLECULAR GENETICS OF THE TCA CYCLE

Gene Localization and Cloning

The genes encoding the enzymes of the TCA cycle are summarized in Table 1. This table includes details for fumarate reductase, which is involved in the anaerobic generation of succinate, and pyruvate dehy-

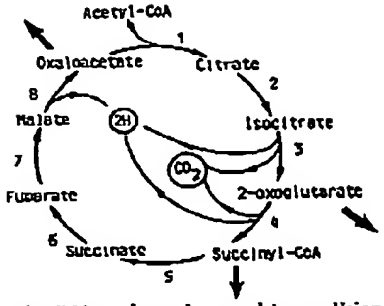

FIG. 1. The TCA cycle under aerobic conditions. The enzymes are as follows. 1, Citrate synthase; 2, aconitase; 3, isocitrate dehydrogenase; 4, 2-oxoglutarate dehydrogenase; 5, succinyl-CoA synthetase; 6, succinate dehydrogenase; 7, fumarase; 8, malate dehydrogenase. Heavy arrows represent fluxes to biosynthesis.

drogenase. Although the latter enzyme is not formally part of the TCA cycle, it shares one subunit with 2-oxoglutarate dehydrogenase, and the control of expression of the two complexes is of considerable interest. All of the genes listed in Table 1 have now been cloned, barring that for aconitase, and the nucleotide sequences of several have been determined. The locations of the genes on the genetic map of *Escherichia coli* are also shown in Table 1.

The most striking feature to emerge from these data is the existence of a cluster of TCA cycle genes at approximately 17 min on the *E. coli* linkage map. Conventional genetic mapping had indicated that the TCA cycle genes were in close proximity to each other but that several other genes were interspersed between them (e.g., reference 3). However, the complete nucleotide sequence of this region has now been established (10, 22, 23, 75, 90, 109), and it is clear that nine TCA cycle genes are in fact contiguous. These genes, *gltA–sdhCDAB–sucABCD*, encode four enzymes or enzyme complexes of the TCA cycle, apparently in three different transcription units. Of the other genes encoding TCA cycle enzymes, that for aconitase has not been located, and those for isocitrate dehydrogenase, fumarase, and malate dehydrogenase are well separated from each other and from the nine-gene cluster. The four genes encoding the components of fumarate reductase are found in a single transcription unit at 94 min on the linkage map (15, 16, 35) (Table 1).

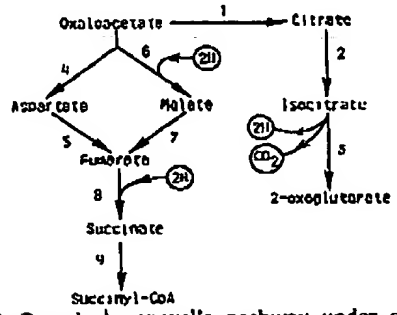

FIG. 2. Branched noncyclic pathway under anaerobic conditions. The enzymes are as follows. 1, Citrate synthase; 2, aconitase; 3, isocitrate dehydrogenase; 4, glutamate-oxaloacetate aminotransferase; 5, aspartase; 6, malate dehydrogenase; 7, fumarase; 8, fumarate reductase; 9, succinyl-CoA synthetase.

TABLE 1. Genes encoding enzymes of the TCA cycle

Enzyme (gene)	Map location[a] (min)
Citrate synthase (*gltA*)	17
Aconitase	Not known
Isocitrate dehydrogenase (*icd*)	25
2-Oxoglutarate dehydrogenase complex	
2-Oxoglutarate dehydrogenase (*sucA*)	17
Dihydrolipoamide succinyltransferase (*sucB*)	17
Lipoamide dehydrogenase (*lpd*)	3
Succinyl-CoA synthetase	
α Subunit (*sucD*)	17
β Subunit (*sucC*)	17
Succinate dehydrogenase	
Flavoprotein (*sdhA*)	17
Iron-sulfur protein (*sdhB*)	17
Membrane anchor protein (*sdhC*)	17
Membrane anchor protein (*sdhD*)	17
Fumarase (*fumC*[b])	35.5
Malate dehydrogenase (*mdh*)	70
Pyruvate dehydrogenase complex	
Pyruvate dehydrogenase (*aceE*)	3
Dihydrolipoamide acetyltransferase (*aceF*)	3
Lipoamide dehydrogenase (*lpd*)	3
Fumarate reductase	
Flavoprotein (*frdA*)	94
Iron-sulfur protein (*frdB*)	94
Membrane anchor protein (*frdC*)	94
Membrane anchor protein (*frdD*)	94

[a] Data taken from reference 3, except *fumC* (41).

[b] For the possible roles of *fumA* and *fumB*, see Gene Localization and Cloning.

The detailed molecular analysis of the TCA cycle gene cluster is largely the work of Guest and his colleagues. In 1981, Guest (38) selected recombinant plasmids from the Clarke-Carbon gene bank (14) that carried the citrate synthase *gltA* gene by complementation of a *gltA* mutant. Subsequently, the *gltA* gene was subcloned into phage vectors by in vitro recombination, and this segment of DNA was extended by prophage integration and aberrant excision. Derivatives carrying the *sdh* genes, *sucA*, and *sucB* were identified by transduction and complementation of appropriate mutants (91). Finally, subcloning of the region beyond *sucB* revealed the presence of two further genes, *sucC* and *sucD*, which encode, respectively, the β and α subunits of succinyl-CoA synthetase (10). The analysis of this cluster is discussed further below.

The gene *mdh*, encoding malate dehydrogenase, has been cloned by Sutherland and McAlister-Henn (97). Three plasmids in the Clarke-Carbon gene bank (14) were known to contain inserts originating close to the *mdh* gene, but only one directed overexpression of malate dehydrogenase activity. Subsequently, the *mdh* gene was identified and then subcloned from this

Section B. Class II Reactions: Conversion of Precursor Metabolites to Small-Molecule Building Blocks

B1. Biosynthesis of Amino Acids

20. Ammonia Assimilation and the Biosynthesis of Glutamine, Glutamate, Aspartate, Asparagine, L-Alanine, and D-Alanine

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ASSIMILATION OF AMMONIA

Ammonia as Nitrogen Source

Ammonia is the preferred source of nitrogen for the growth of enteric bacteria in a defined minimal medium with glucose as the source of carbon. Although there has been no systematic study of the growth rate of *Escherichia coli* or *Salmonella typhimurium* with various nitrogen sources, we are not aware of any data in the literature or any observation from our laboratory that suggests that any other nitrogen source supports a faster growth rate than ammonia. It is the purpose of this section to describe both how ammonia is assimilated and the relationship between ammonia assimilation and the biosynthesis of glutamate and glutamine in the enteric bacteria, *E. coli* and *S. typhimurium*. Information gained from the study of the related enteric organism, *Klebsiella aerogenes*, has sometimes complemented that from *E. coli* and *S. typhimurium*; therefore, *K. aerogenes* is discussed when appropriate. This subject has been most recently reviewed by Tyler (123) and Magasanik (74).

All cellular nitrogen for the synthesis of macromolecules in the enteric bacteria is derived from the amido group of glutamine, the amino group of glutamate, or directly from incorporation of ammonia. Glutamate provides nitrogen for the synthesis of most of the amino acids, whereas glutamine donates nitrogen for the synthesis of purines, pyrimidines, amino sugars, histidine, tryptophan, asparagine, NAD, and *p*-aminobenzoate. A kilogram of dry weight of *E. coli*

contains 11 to 12 g-atoms of total nitrogen; the synthesis of glutamate and its products requires about 10 g-atoms of nitrogen, whereas the synthesis of glutamine and nitrogen-containing compounds which derive nitrogen from the glutamine amide requires 1.3 g-atoms of nitrogen (144). When the ammonium ion concentration of the growth medium is sufficiently high (greater than 1 mM), ammonia is incorporated directly into glutamate, glutamine, and asparagine. However, when the ammonium ion concentration of the growth medium is less than about 0.1 mM, ammonia is incorporated into glutamine only.

The reactions responsible for ammonia assimilation and the synthesis of glutamate and glutamine in ammonia-containing medium are shown below.

$$NH_3 + \text{glutamate} + ATP \xrightarrow[Mg^{2+}]{} \text{glutamine} + ADP + P_i \quad (1)$$

$$\text{Glutamine} + \text{2-keroglutarate} + NADPH \rightarrow 2\ \text{glutamate} + NADP^+ \quad (2)$$

$$NH_3 + \text{2-ketoglutarate} + NADPH \rightarrow \text{glutamate} + NADP^+ \quad (3)$$

These three reactions are catalyzed by glutamine synthetase, glutamate synthase, and glutamate dehydrogenase, respectively

The reaction catalyzed by glutamine synthetase is the only known biosynthetic route for the synthesis of glutamine. Mutations in *glnA*, the structural gene for glutamine synthetase, result in an absolute requirement for glutamine; thus, the *glnA* gene is the only gene coding for a glutamine synthetase (66, 74, 82). A strain with mutations that result in the loss of both glutamate dehydrogenase and glutamate synthase is a glutamate auxotroph. Thus, the enzymes essential for ammonia assimilation and for the synthesis of glutamine and glutamate are glutamine synthetase, glutamate synthase, and glutamate dehydrogenase.

A strain devoid of glutamate dehydrogenase activity (equation 3) has no detectable phenotype, but a deficiency of glutamate synthase (equation 2) results in the inability to grow when the medium contains a low level of ammonium ion or a nitrogen source which generates ammonia slowly. These observations were made first in *Klebsiella* species and subsequently in *E. coli* and *S. typhimurium* (8, 13, 14, 31, 92, 98). When cells are grown in ammonia-containing medium, both glutamate dehydrogenase and glutamate synthase synthesize glutamate. For cells in medium with a growth rate-limiting source of ammonia, glutamate dehydrogenase is not involved in ammonia assimilation and glutamate formation; instead, glutamine synthetase (equation 1) is the only active ammonia-assimilating enzyme, and glutamate synthase (equation 2) is the only active glutamate-forming enzyme. Therefore, glutamine synthetase has two functions: the synthesis of glutamine and the assimilation of ammonia when the growth of the cell is limited by the availability of ammonia.

About eight times more glutamate than glutamine is required for cellular biosyntheses; consequently, during growth in a medium containing a low level of ammonia or some other source of nitrogen, when ammonia is assimilated exclusively into glutamine by glutamine synthetase, most of the glutamine must be recycled to glutamate. This means that the glutamine-dependent amidotransferases other than glutamate synthase which convert glutamine to glutamate can only provide about 12% of the cellular glutamate during ammonia-limited growth. Apparently, glutamate synthase produces the remaining 88% of the cellular glutamate.

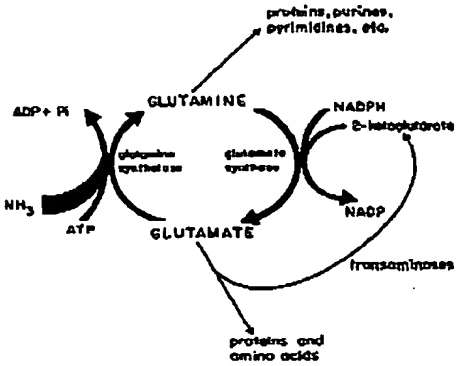

FIG. 1. Ammonia assimilatory cycle.

The reactions catalyzed by glutamine synthetase and glutamate synthase form an ammonia assimilatory cycle (Fig. 1). This cycle allows the net assimilation of ammonia into glutamine via glutamine synthetase and the replenishment and maintenance of an adequate intracellular level of glutamate.

Sources of Nitrogen Other than Ammonia

E. coli and *S. typhimurium* can grow on a variety of organic nitrogen-containing compounds as sole source of nitrogen but cannot grow on any inorganic nitrogen compound except ammonia. However, the related *Klebsiella* species can utilize nitrate, nitrite, or atmospheric diatomic nitrogen. A limited number of organic nitrogen compounds, about 25, can support growth as sole sources of nitrogen. The best surveys are presented by Gutnick et al. (54) for *S. typhimurium* and by Tyler (132) for *E. coli*. Brenchley et al. (15), Chelbe et al. (114, 115), and Wild et al. (139) sometimes used nitrogen sources that were not included in the surveys. Therefore, the surveys should be considered incomplete and probably strain dependent. Furthermore, variants of *E. coli* have been isolated that can grow on an extended range of nitrogen sources (63).

Growth with these organic nitrogen sources is invariably slower than with ammonia. We term such growth nitrogen limited. Both the level and the specific activity of glutamine synthetase are invariably high, consistent with its role in ammonia assimilation. This observation strongly indicates that the growth-limiting factor is always the rate of ammonia generation from these nitrogen sources and of its subsequent assimilation. The same regulatory system that regulates the level and activity of glutamine synthetase also regulates the level of an ammonia uptake system, thereby increasing the ability of the cell to scavenge a diminishing supply of ammonia (58).

The degradative enzymes and transport systems for a number of these nitrogen sources are frequently induced by growth in a nitrogen-limited medium. These systems, termed Ntr for nitrogen regulated, are involved in the degradation of arginine, ornithine, agmatine, putrescine, and γ-aminobutyrate in *E. coli* (115, 146); the degradation of histidine and urease in *K. aerogenes* (123); the transport of γ-aminobutyrate and glutamine in *E. coli* (59, 142); and the transport of glutamine, arginine, aspartate, lysine, ornithine, glutamate, and histidine in *S. typhimurium* (44, 65). The regulation of the *hisJQMP* and *argT* operons in *S. typhimurium*, which specify components of the histidine and arginine transport systems, was shown to be transcriptional (120). In the most extensive study of the transport systems of amino acids, Kustu et al. (65) showed increased levels of many periplasmic binding proteins that are involved in amino acid transport in *S. typhimurium*. This is the only report that correlates increased transport activity during nitrogen deprivation with the presence of specific proteins.

The same regulatory system controls the activity and level of glutamine synthetase and all of the Ntr systems. The activation during nitrogen-limited growth is mediated by two regulatory proteins, P_{II} and the bifunctional uridylyltransferase/uridylyl-removing enzyme, the activities of which are determined by the intracellular ratio of glutamine/2-ketoglutarate, a sensitive barometer of the ammonia content of the environment. The transcriptional regulation of *glnA* expression and the regulation of glutamine synthetase activity are discussed under Glutamine, below. In summary, nitrogen deprivation elevates the activities of a number of proteins that allow the bacteria to respond to an environmental stress in a highly coordinated fashion.

There are two general classes of nitrogen sources. The first class of compounds, when degraded, generate ammonia; D- and L-serine are members of this class. The second class consists of compounds that are degraded to glutamate, such as proline or aspartate, or that can form glutamate through transamination, such as 2-aminobutyrate. Some amino acids generate both glutamate and ammonia when degraded.

Growth with an ammonia-generating nitrogen source requires a high level of glutamine synthetase for ammonia assimilation and of glutamate synthase for replenishment and synthesis of the glutamate. A strain deficient in glutamate synthase cannot grow on any of the ammonia-generating nitrogen sources except D-serine (98, 123). Apparently, D-serine is degraded so rapidly that enough ammonia is generated for the synthesis of glutamate by glutamate dehydrogenase, which obviates the need for glutamate synthase.

Growth with a glutamate-generating nitrogen source also requires a high level of glutamine synthetase for the utilization of the ammonia derived from glutamate by an unidentified pathway which utilizes neither glutamate dehydrogenase nor aspartase (Reitzer and Magasanik, unpublished data). Paradoxically, a *gltB* mutant, which is deficient in glutamate synthase, cannot grow with most of the glutamate-generating nitrogen sources, such as arginine or proline (98, 123). This is an interesting consequence of the fact that much of the glutamine produced from the assimilation of ammonia must be converted back to glutamate by glutamate synthase. When *gltB* strains are grown with arginine or proline, the rate of ammonia generation limits growth. The ammonia is assimilated by glutamine synthetase to form glutamine and is not converted to glutamate. The accumulation of glutamine represses both glutamine synthetase and other Ntr systems (see Glutamine, below). Arginine is transported and degraded by Ntr systems, but because glutamine accumulates, these systems are repressed and the cell cannot accumulate or degrade the arginine. Genetic suppression of the inability of *gltB* cells to grow with a glutamate-forming source of nitrogen results in the constitutively high synthesis of glutamine synthetase and the Ntr systems (98, 123). Once the glutamate-forming nitrogen source is degraded, the need for glutamate synthesis by glutamate synthase is obviated. Furthermore, growth of a wild-type strain of *S. typhimurium*, with arginine as a nitrogen source, represses glutamate synthase. In other words, glutamate synthase is required for the establishment, but not the maintenance, of steady-state nitrogen-limited growth with a glutamate-forming source of nitrogen. The fact that a *gltB* strain cannot grow on a particular glutamate-forming nitrogen source has been taken to imply that the degradation of that nitrogen source is regulated by nitrogen deprivation (123). A *gltB* strain can grow with glutamate, aspartate, and asparagine as source of nitrogen. This probably means that the degradation of these compounds does not require an Ntr system.

Growth with L-glutamine as the nitrogen source sustains a growth rate almost as rapid as growth with ammonia, but, curiously, the transcription of *glnA* and other Ntr systems is activated, indicating that growth on glutamine is nitrogen limited. It is unexpected that growth with glutamine as the nitrogen source should increase the expression of a gene the product of which synthesizes glutamine. Glutamine per se does not cause any unusual changes in metabolism because cells grown in medium containing both glutamine and ammonia have a low level of glutamine synthetase (74). The glutamate synthase appears to be sufficiently active to deplete the intracellular glutamine pool when cells are grown with glutamine as sole source of nitrogen. This view is supported by the observation that, in a *gltB* strain, growth with L-glutamine as the nitrogen source results in a low level of glutamine synthetase, in consequence of the intracellular accumulation of glutamine (14). The significance of this metabolic oddity is that glutamine-requiring strains and some strains deficient in their ammonia assimilatory capacity can be grown on a nitrogen-limited medium. This has greatly facilitated the study of the response of *E. coli* and *S. typhimurium* to nitrogen starvation.

Pleiotropic Defects in Nitrogen Utilization

Mutations in genes that code for proteins other than glutamine synthetase, glutamate synthetase, or their regulatory proteins can cause a pleiotropic inability to grow with a variety of single nitrogen sources other than ammonia. *E. coli* and *K. aerogenes* have two asparagine synthetases, an ammonia-dependent enzyme and a glutamine-dependent enzyme. A deficiency of the glutamine-dependent enzyme in *K. aero-*

Regulation of Glutamine Synthetase by Cumulative Feedback Inhibition

Glutamine synthetase has two functions: the formation of glutamine for the synthesis of protein and other nitrogen compounds and the assimilation of ammonia when the availability of ammonia in the environment is restricted. In an ammonia-rich medium, the level of glutamine synthetase is low, and glutamine synthetase functions primarily for the synthesis of glutamine, whereas in an ammonia-poor, nitrogen-limited medium the level of glutamine synthetase is high, and glutamine synthetase has both functions. The ammonia assimilatory function is quantitatively more significant, as is evident from the fact that almost all of the glutamine synthesized must be reconverted to glutamate by glutamate synthase.

The cumulative feedback inhibition of glutamine synthetase affects only adenylylated glutamine synthetase (118). The physiological significance of this inhibition is evident when the functions of glutamine synthetase are considered. When cells are grown in a nitrogen-limited medium, glutamine synthetase functions primarily to assimilate ammonia. Glutamine synthetase is not adenylylated and not susceptible to feedback inhibition. However, when the cells are grown in an ammonia-containing medium, glutamine synthetase is partially adenylylated and functions primarily in the formation of glutamine for the synthesis of protein and in the formation of some nitrogenous intermediates. Therefore, it is appropriate that glutamine synthetase is susceptible to inhibition by the products of glutamine metabolism only in the ammonia-containing medium when glutamine synthetase is not necessary for ammonia assimilation. As a corollary, part of the function of the adenylylation cascade is to make glutamine synthetase susceptible to cumulative feedback inhibition.

GLUTAMATE

Synthesis of Glutamate

Strains lacking both glutamate synthase and glutamate dehydrogenase have an absolute requirement for glutamate (8, 13, 14, 73, 123). The presence of either enzyme in the cell allows the synthesis of glutamate in ammonia-containing minimal medium. Strains deficient in glutamate dehydrogenase have no growth phenotype, whereas cells deficient in glutamate synthetase, an enzyme required for the assimilation of ammonia when it is present in the medium in low concentration (see Assimilation of Ammonia, above), fail to grow with a variety of nitrogen sources. Strains of *E. coli* and *K. aerogenes* lacking glutamate synthase can grow with glutamate, asparagine, aspartate, and D-serine and very slowly with glutamine as sole source of nitrogen (14, 92, 98; for a review, see reference 123). Mutations in strains of *S. typhimurium* causing the complete loss of glutamate synthase activity result in the inability to utilize arginine as sole source of nitrogen, but these strains were not further characterized with regard to their growth with other nitrogen sources (31). As discussed in the previous sections, the inability of glutamate synthase-deficient strains to grow on some amino acids that give rise to glutamate results from their failure to produce the Ntr systems that degrade these compounds. This failure is due to the inability of these mutants to convert the glutamine, formed by ammonia assimilation, to glutamate. Therefore, glutamate synthase has two functions, the synthesis of glutamate and the removal of glutamine, the primary product of ammonia assimilation during nitrogen-limited growth.

Glutamate Synthase

Properties of the purified enzyme. In 1970, Tempest, Meers, and Brown (84, 121) discovered glutamate synthase, which catalyzes the reductive amination (NADPH dependent) of 2-ketoglutarate with glutamine as the nitrogen donor. They suspected the existence of a route of glutamate synthesis independent of glutamate dehydrogenase, because during the growth of *K. aerogenes* in a nitrogen-limited medium, glutamate dehydrogenase was repressed, but the intracellular pool of glutamate was normal. The discovery of glutamate synthase established a previously unknown pathway of glutamate formation from ammonia: first, ammonia is incorporated by glutamine synthetase into glutamine, and then the amide of glutamine is transferred to 2-ketoglutarate to form two molecules of glutamate.

The enzyme was subsequently purified from *E. coli* W and *K. aerogenes*. The enzyme has two nonidentical subunits in equimolar amounts. In *E. coli*, the subunits have molecular weights of 53,000 and 135,000; in *K. aerogenes*, the subunits have molecular weights of 51,500 and 175,000 (88, 122). The native molecular weight of the purified enzyme of *E. coli* was estimated to be 800,000 by sedimentation equilibrium and gel filtration; the enzyme in *K. aerogenes* had an $s_{20,w}$ consistent with this molecular weight. However, Miller and Stadtman (88) noticed that the molecular weight (the $s_{20,w}$) was 13S instead of 20S after an early stage of the purification. Furthermore, Sakamoto et al. (112) showed that active glutamate synthase had two molecular weights, 200,000 and 800,000, and two $s_{20,w}$s, 13S and 20S. Therefore, active glutamate synthase is probably a dimer that aggregates during the course of purification. Glutamate synthase has not been purified from *S. typhimurium*, but antiserum raised against *E. coli* enzyme precipitates two polypeptides from *S. typhimurium* with the same molecular weights as those from *E. coli* (73).

The purified enzyme contains flavin (both flavin adenine dinucleotide and flavin mononucleotide), iron (mostly ferrous), and labile sulfide. A stoichiometry of 1:4:4 was suggested from the data of Miller and Stadtman (88) and from observations in other iron-sulfide-containing flavoproteins. This suggested stoichiometry is at some variance with the data of Trotta et al. (122). The large subunit of the *K. aerogenes* enzyme was associated with the flavin and the iron-sulfide (122). Flavin adenine dinucleotide, not flavin mononucleotide, has been implicated as the active species of flavin, but the data presented were considered inconclusive (88).

Many glutamine-dependent amidotransferases, including glutamate synthase, hydrolyze glutamine to glutamate and ammonia without the transfer of the amide to the appropriate substrate. For a thorough

discussion of amidotransferases, the reader should consult the excellent review of Buchanan (16). The glutaminase activity of purified glutamate synthase can be 10% of the glutamate synthetic activity in *E. coli* and *K. aerogenes* (50, 75, 122). Two lines of evidence indicate that the glutaminase activity is a property of the larger subunit. First, affinity labeling (alkylation) with L-2-amino-4-oxo-5-chloropentanoic acid (chloroketone) binds the large subunit and inactivates both the glutaminase and the glutamate synthase activity (76, 122). Second, the large subunit, separated from the small subunit, has the glutaminase activity (75). The glutaminase activity is not affected by the removal of the flavin, a finding that implies that the flavin is not required for the binding of glutamine (75). Storage at high pH increases the glutaminase activity, a result that has been seen with another amidotransferase, carbamyl phosphate synthetase, and has been taken to imply that the glutaminase activity is evident only after the enzyme has sustained some form of damage (50, 135). Several researchers have commented on the uniqueness of glutamate synthase because the glutamine-binding subunit is the larger of the two subunits. It should be noted that the size of the small subunit of glutamate synthase is not unusual for the large subunit of many amidotransferases (16). Therefore, the large subunit of glutamate synthase is atypically large for the glutamine-binding subunit. This probably results from the fact (described below) that this subunit also participates in electron transfer, which is unusual for an amidotransferase.

Glutamate synthase and other amidotransferases can utilize a high level of ammonia in place of glutamine as nitrogen donor. The rate of the ammonia-dependent reaction depends on the organism that serves as the source of glutamate synthase; the ratio of the ammonia-dependent activity to the glutamine-dependent activity is 10 and 1.4% when the enzyme is from *K. aerogenes* and *E. coli* B, respectively (50, 122). The ammonia-dependent activity is a property of the small subunit by a number of criteria. First, a variety of treatments that inactivate or damage the large subunit have no effect on the ammonia-dependent activity. These treatments include alkylation by the glutamine analog, chloroketone, and removal of the flavin, the iron-sulfide, or both from the large subunit (50, 75–77, 122). Second, the small subunit alone can catalyze the ammonia-dependent reaction (75). It should be noted that the ammonia-dependent reaction is the same reaction as that catalyzed by glutamate dehydrogenase. The available evidence suggests that this activity is not the result of contamination by glutamate dehydrogenase. First, Mantsala and Zalkin (77) were unable to detect glutamate dehydrogenase in their glutamate synthase preparation by a variety of immunological methods. Second, preincubation of purified glutamate synthase at low pH stimulates the ammonia-dependent activity of glutamate synthase but destroys the activity of purified glutamate dehydrogenase (77).

Miller and Stadtman (88) first proposed that the reaction catalyzed by glutamate synthase occurs in two steps: the reduction of the enzyme-bound flavin by NADPH, followed by the reaction of the reduced flavin with 2-ketoglutarate and glutamine to generate oxidized flavin and two glutamates. This view has been supported by all subsequent data. After the chemical (nonenzymatic) reduction of the flavin by dithionate, the flavin is reoxidized by the substrate glutamine and 2-ketoglutarate with the formation of glutamate (50, 77, 88). The iron-sulfur cluster also appears to be involved in the electron transfer (unpublished observation cited in reference 107). As noted before, removal of the flavin and iron does not inhibit the ammonia-dependent reaction but does abolish the glutamine-dependent reaction (50, 76, 77). Furthermore, in the ammonia-dependent reaction, the electrons are transferred directly to 2-ketoglutarate, whereas in the glutamine-dependent reaction they are transferred to water (50). From these results, it has been concluded that the electron transport for the ammonia-dependent reaction occurs by a nonphysiological route.

Kinetic data have been obtained from the enzymes purified from *E. coli* and *K. aerogenes*. The K_ms for glutamine and NADPH are of the order of 230 to 300 μM and 2.2 to 12 μM, respectively. There is a large discrepancy in the K_ms for 2-ketoglutarate; the *E. coli* enzyme has a low K_m, about 5 μM, whereas the *K. aerogenes* enzyme has a K_m of 300 μM. This difference may be the only manifestation of the different-sized large subunits formed by these bacteria. The reader is referred to the references for more kinetic, physiochemical, and enzymological information (11, 76, 88, 107, 122).

From these data, the following scheme for the overall glutamate synthase reaction can be proposed. NADPH binds the small subunit and transfers electrons to the large subunit, which reduces the flavin. 2-Ketoglutarate binds the small subunit, and glutamine binds the large subunit. The glutamine amide is transferred to 2-ketoglutarate, and the reduced flavin reduces a proposed iminoglutarate intermediate to glutamate. Clearly, the loss of either subunit by mutation should result in the inability to catalyze the glutamate synthase reaction.

Characterization of the genes coding for the subunits of glutamate synthase. The mutations that result in the loss of glutamate synthase have been located in the three enteric species and are located at a position corresponding to 69 min on the *E. coli* chromosome (43, 46, 98). Only one locus has been found for mutations that result in the loss of glutamate synthase. This observation suggests that the genes for the two subunits are linked. This locus has been identified as specifying the structural genes in *S. typhimurium* (43). The locus for glutamate synthase has been designated *gltB* in earlier publications; however, recently the designations *gltB* and *gltD* have been proposed for the large and small subunits, respectively (73).

The two genes are part of one transcriptional unit and consititute the *gltBD* operon (49, 72, 73). The glutamate synthase genes were cloned from *E. coli*, and the hybrid ColE1 plasmid overproduced glutamate synthase threefold (29). Using antiserum raised against purified glutamate synthase from *E. coli*, Lozoya et al. (72) showed that this plasmid directed the synthesis of both subunits of glutamate synthase. A deletion analysis of plasmid-borne glutamate synthase and the analysis of polar insertion mutations indicate that both subunits are translated from one

transcript and that the large subunit is transcribed first (49, 73). The original mutant strain of *E. coli* analyzed by Berberich (8) failed to synthesize either subunit and probably carries a polar mutation in *gltB* (72). Madonna et al. (73) showed in *S. typhimurium* that loss of only the large subunit is sufficient to cause the characteristic phenotype of *gltB* mutants. Furthermore, a *gltB gdh* strain of *S. typhimurium*, which has the small subunit of glutamate synthase but not the large subunit or glutamate dehydrogenase, requires glutamate for growth (73); therefore, despite the observation that the purified small subunit has glutamate dehydrogenase-like activity, this activity does not provide the intact cell with glutamate.

Regulation of glutamate synthase. The primary form of regulation appears to be repression by glutamate or activation by glutamate deprivation, although very little is known about the mechanism of regulation. Despite the important role of glutamate synthase in the assimilation of ammonia during growth in an ammonia-restricted medium, the level of glutamate synthase does not correlate with the level of glutamine synthetase, the other enzyme required for ammonia assimilation. The level of glutamate synthase is generally highest in ammonia-containing minimal medium. Restriction of ammonia availability results in no change in activity in *E. coli*, slightly higher activity in *S. typhimurium*, and slightly lower activity in *K. aerogenes* (12, 14, 88). In all three bacterial species, glutamate synthase is repressed when the sole source of nitrogen is degraded to or provides glutamate; these nitrogen sources are glutamate, arginine, glutamine, histidine, and proline (6, 12, 14, 88, 109). When ammonia is added to these media, the glutamate synthase is not repressed (6, 12). The presence of ammonia causes repression of the Ntr systems that degrade these nitrogen sources, and the major source of nitrogen for glutamate is then ammonia. Aspartate, which is readily transaminated to glutamate, apparently can rapidly enter *S. typhimurium* in the presence of ammonia because aspartate in the medium can repress glutamate synthase even in the presence of ammonia (12). Glutamate synthase in *K. aerogenes* is also repressed when histidine is the sole source of carbon and nitrogen; histidine is degraded to glutamate (6). It should be noted that this medium is considered nitrogen rich and carbon poor. To summarize these observations, there is no observation inconsistent with the hypothesis that glutamate represses glutamate synthase. The lack of repression by some glutamate-producing nitrogen sources in ammonia-containing medium results from the failure to form the enzymes responsible for their degradation. Glutamate synthase can be repressed in either nitrogen-limited or nitrogen-rich medium, an observation that implies that there is no relation of necessity between the control of synthesis of glutamate synthase and that of glutamine synthetase.

There have been a few observations that suggest that tRNAs may be involved in glutamate synthase regulation. Lapointe et al. (69) showed that, in an *E. coli* strain with a temperature-sensitive glutamyl-tRNA synthetase, the levels of glutamate synthase and glutamine synthetase were 10 times higher than those in the wild-type strain. The strains had been grown in broth medium, which is highly repressive for both enzymes (12). The elevated level of glutamine synthase could be the result of the elevated glutamate synthase level, which could deplete the intracellular glutamine. The elevated level of glutamate synthase may result from an increased level of uncharged glutamyl-tRNA, although no mechanism linking tRNA charging to glutamate synthase regulation has been proposed. Rosenfeld and Brenchley (109) have shown that, in *hisT* strains of *S. typhimurium*, which have an altered pseudouridine synthetase I, resulting in undermodification of two uridines in many anticodon loops of tRNA, the activity of glutamate synthase is decreased twofold in glucose-ammonia (nitrogen-excess) and glucose-arginine (nitrogen-limiting) medium; however, the glutamate synthase activity was not altered in a different nitrogen-limiting medium. The *hisT* strains do not have an observable defect in ammonia assimilation and, in fact, grow faster on some nitrogen sources. Considering the potential pleiotropic nature of such mutations which affect macromolecular synthesis and which could result in the alterations of metabolic pools, it is difficult to make a strong case for the direct involvement of a glutamyl-tRNA in the regulation of glutamate synthase (24).

Glutamate Dehydrogenase

Glutamate dehydrogenase is a completely dispensable enzyme; a strain deficient in glutamate dehydrogenase has no observable growth phenotype (13, 123, 129). Mutants with lesions in *gltB* do not require glutamate if the ammonium ion concentration in the medium is greater than about 1 mM, indicating that glutamate dehydrogenase can synthesize glutamate when provided with sufficient ammonia (123).

Glutamate dehydrogenase has been purified from *E. coli* and *S. typhimurium*. The purified enzyme can use NADPH, but not NADH, for the reduction of 2-ketoglutarate, an observation that suggests a biosynthetic function. The enzyme is a hexamer of identical subunits with a molecular weight of 300,000. The K_ms for ammonia and 2-ketoglutarate are about 1 mM (27, 28, 112, 130).

Mutations resulting in the loss of glutamate dehydrogenase have been mapped to a site located at 27 min on the *E. coli* and *S. typhimurium* chromosomes (6, 98, 110); in *S. typhimurium*, this was shown to be the locus for the structural gene (110). The gene for glutamate dehydrogenase, which has been designated *gdh* (or *gdhA*), *gdhD*, and *gdhA* in *E. coli*, *K. aerogenes*, and *S. typhimurium*, respectively, has been cloned from all three organisms (29, 79, 143). The sequence of the amino terminus of glutamate dehydrogenase from *K. aerogenes* and the complete sequence from the *E. coli* enzyme have been determined (83, 89, 126, 127). There is a high degree of homology between the glutamate dehydrogenases from the enteric bacteria and, surprisingly, enzymes in *Neurospora crassa* and *Saccharomyces cerevisiae* (79, 87, 89, 127).

Knowledge of the start sites for transcription and the sequences for the promoters would be of interest because the regulation of glutamate dehydrogenase differs in enteric bacteria. However, promoters have not been identified beyond sequence homology to other promoters. The glutamate dehydrogenase activity is repressed in *E. coli* by the presence of glutamate

in the medium; there is no control by the availability of ammonia in the medium (128). Restricting the extent of the charging of glutamyl-tRNAs does not affect the level of glutamate dehydrogenase, an observation that suggests that glutamyl-tRNAs do not play a role in repression (69). In *K. aerogenes*, growth in a nitrogen-limited medium represses glutamate dehydrogenase, even when exogenous glutamate is not provided (12, 14). The intracellular pool of glutamate is relatively invariant when cells of *K. aerogenes* are grown with an excess or limiting amount of ammonium sulfate (84); therefore, the glutamate pool does not regulate glutamate dehydrogenase in *K. aerogenes*. In *S. typhimurium*, glutamate dehydrogenase is not regulated by the quality of the nitrogen source or the presence of exogenous glutamate. The only regulation seen is when cells are grown in rich broth medium or with a mixture of amino acids (12).

The inverse correlation between the degree of nitrogen starvation and the level of glutamate dehydrogenase activity in *K. aerogenes* has been studied by Bender et al. (6, 7). Certain mutations in *glnL* (*glnL45*) cause constitutively high expression of *glnA* specifying glutamine synthetase and *glnG* specifying NR_I and result in repression of glutamate dehydrogenase. A strain with *glnL45* and *gltB* mutations requires glutamate for growth because glutamate synthase activity is deficient and glutamate dehydrogenase is repressed. One class of revertant strains, selected for the ability to grow on glucose-ammonia, had nonrepressible glutamate dehydrogenase activity but also failed to produce histidase (an Ntr system) during nitrogen limitation (6, 7). A mutation in a previously unidentified gene, *nac* (nitrogen assimilatory control), was identified as being responsible for this phenotype. It is possible that the *nac* gene product plays a role in the response of certain genes to nitrogen limitation and that it specifically acts as a repressor *gdhD*, the structural gene for glutamate dehydrogenase.

ASPARTATE

Aspartate is synthesized as follows from oxaloacetate by transamination with glutamate as the amino donor: oxaloacetate + L-glutamate → L-aspartate + 2-ketoglutarate. This is probably the universal route of its synthesis. Virtually all of the biochemical and genetic studies of aspartate biosynthesis have been made in *E. coli*; it would be surprising if the synthesis of aspartate differed substantially in *S. typhimurium*. Aspartate synthesis has been reviewed most recently by Umbarger (125) and Reitzer (102).

Bacterial transaminases were first studied in *E. coli* by Rudman and Meister (111). They found three general transaminases which could donate the amino group of a variety of amino acids to 2-ketoglutarate to form glutamate. This is the reverse reaction of the transaminase in the growing cell. Some researchers still assay transaminase in this reverse direction, which has caused confusion in the literature. Rudman and Meister (111) designated these activities transaminase A for aspartate, tryptophan, tyrosine, and phenylalanine; transaminase B for leucine, isoleucine, and valine; and transaminase C for the following reaction: L-valine + pyruvate → 2-ketoisovalerate + L-alanine.

Subsequent work has shown that transaminase A is really two enzymes and that they are the only two enzymes that synthesize aspartate. One enzyme is the major aspartate transaminase. It has a low K_m for oxaloacetate, 0.4 mM, and a high K_m for the 2-keto analogs of phenylalanine and tyrosine. The activity of this component of transaminase A is constitutive (26, 52, 80). The second component of transaminase A is tyrosine repressible and has a low K_m for the 2-keto acid analogs of phenylalanine and tyrosine. The K_m of the tyrosine-repressible component for oxaloacetate is high, 3 mM (22, 23, 26, 80, 81, 116, 131). The predominant activity can be considered the high-affinity aspartate transaminase, and the tyrosine-repressible enzyme can be considered the low-affinity aspartate transaminase. There is confusion in the literature as to the nomenclature of these enzymes. We suggest that the high-affinity enzyme be called transaminase A1 and that the low-affinity enzyme be called transaminase A2.

Both aspartate transaminases have been purified to homogeneity. Both are dimers with identical subunits; the molecular weights are 82,000 and 88,000 for the high- and low-affinity aspartate transaminases, respectively (80, 81). The amino acid sequence of the aspartate transaminase A1 from *E. coli* B has been completely determined by Kondo et al. (61). The subunit is composed of 396 amino acids and has a molecular weight of 43,573. The *E. coli* enzyme is 40% homologous to the pig heart isozymes.

There has been only one genetic study of aspartate auxotrophy. A specific requirement for aspartate in ammonia-containing minimal medium results from mutations in both *aspC* and *tyrB*, which cause the loss of transaminases A1 and A2. The double mutant requires aspartate and tyrosine for growth. A look at the transaminases characterized by Rudman and Meister (111) would suggest that a strain deficient in transaminase A would also require phenylalanine for growth. However, transaminase B, the product of the *ilvE* gene, also participates in the synthesis of phenylalanine. Neither an *aspC*$^+$ *tyrB* strain nor an *aspC* *tyrB*$^+$ strain requires any amino acid for growth, but the latter strain produces small colonies on minimal medium agar plates. Thus, it may be that the *aspC* gene product, the high-affinity aspartate transaminase, is the predominant aspartate transaminase (52). Two groups have isolated mutants that have lost transaminase A1. The mutations result in the loss of a 44,000-dalton polypeptide, but the mutations were located at different positions on the *E. coli* chromosome (51, 78). Therefore, the structural gene for the high-affinity aspartate transaminase has not yet been identified. It is curious that aspartate cannot be synthesized by a reversal of the following reaction catalyzed by aspartase: aspartate → fumarate + ammonia. The reaction is readily reversible, as is apparent from the fact that the commercial production of aspartate involves the addition of fumarate and ammonia to immobilized cells of *E. coli* (145). However, an aspartate auxotroph is deficient in the two components of transaminase A, not in aspartase. Therefore, aspartate cannot be synthesized by a reversal of aspartase. It is possible that the intracellular concentration of fumarate is too low for aspartate synthesis during growth in ammonia-containing minimal medium, unless fumarate is provided exogenously.

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Metabolic Flux Ratio Analysis of Genetic and Environmental Modulations of *Escherichia coli* Central Carbon Metabolism

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The response of *Escherichia coli* central carbon metabolism to genetic and environmental manipulation has been studied by use of a recently developed methodology for metabolic flux ratio (METAFoR) analysis; this methodology can also directly reveal active metabolic pathways. Generation of fluxome data arrays by use of the METAFoR approach is based on two-dimensional ^{13}C-^{1}H correlation nuclear magnetic resonance spectroscopy with fractionally labeled biomass and, in contrast to metabolic flux analysis, does not require measurements of extracellular substrate and metabolite concentrations. METAFoR analyses of *E. coli* strains that moderately overexpress phosphofructokinase, pyruvate kinase, pyruvate decarboxylase, or alcohol dehydrogenase revealed that only a few flux ratios change in concert with the overexpression of these enzymes. Disruption of both pyruvate kinase isoenzymes resulted in altered flux ratios for reactions connecting the phosphoenolpyruvate (PEP) and pyruvate pools but did not significantly alter central metabolism. These data indicate remarkable robustness and rigidity in central carbon metabolism in the presence of genetic variation. More significant physiological changes and flux ratio differences were seen in response to altered environmental conditions. For example, in ammonia-limited chemostat cultures, compared to glucose-limited chemostat cultures, a reduced fraction of PEP molecules was derived through at least one transketolase reaction, and there was a higher relative contribution of anaplerotic PEP carboxylation than of the tricarboxylic acid (TCA) cycle for oxaloacetate synthesis. These two parameters also showed significant variation between aerobic and anaerobic batch cultures. Finally, two reactions catalyzed by PEP carboxykinase and malic enzyme were identified by METAFoR analysis; these had previously been considered absent in *E. coli* cells grown in glucose-containing media. Backward flux from the TCA cycle to glycolysis, as indicated by significant activity of PEP carboxykinase, was found only in glucose-limited chemostat culture, demonstrating that control of this futile cycle activity is relaxed under severe glucose limitation.

Access to complete genome sequence information for a number of microorganisms now motivates the development and application of experimental techniques for phenotype characterization (such as transcriptome and proteome analyses), providing arrays of data that can be directly mapped to corresponding arrays of genes (14, 36). The physiological counterpart to such composition arrays is the array of fluxes (reaction rates on a per-unit cell volume or per-unit cell mass basis) for all of the reactions that occur in the organism, for which we use, by analogy, the term fluxome. Approximate fluxome sets for certain subsets of metabolism can be attained by methods of metabolic flux analysis, which require data on uptake and efflux rates of certain metabolites outside the cell and which assume a corresponding network of metabolic pathways in the cell (39). Alternatively, by use of more recently introduced methodology based on isotopic imprinting of amino acids by their precursors, the active central carbon pathways and the ratios of their fluxes can be directly determined from two-dimensional (2D) nuclear magnetic resonance (NMR) analysis of hydrolyzed cell protein (30–33). This method, for which we introduce the term METAFoR (metabolic flux ratio) analysis, offers a relatively high throughput access to these key fluxome elements, enabling physiological data arrays to be acquired over a broad range of genetic and environmental conditions.

Specifically, METAFoR analysis quantifies the relative abundance of intact carbon bonds originating from uniformly isotopically labeled source molecules by use of proton-detected 2D ^{13}C-^{1}H correlation NMR spectroscopy (COSY) (30, 34, 42). Such 2D NMR analysis of amino acids obtained from hydrolyzed cell protein permits quantitative analysis of the relative abundance of intact, contiguous fragments in the precursor metabolites of central metabolism, because the carbon backbone of these molecules is conserved in the amino acids. Typically, fractional ^{13}C labeling of amino acids is achieved by growing cells with a mixture of 85 to 90% natural-abundance glucose and 10 to 15% [U-$^{13}C_6$]glucose (22, 27, 30–32, 34). Because alternative pathways leading to common intermediates or products produce different intact fragments originating from a single glucose source molecule (30–32), specific multiplet patterns in the ^{13}C fine structures that reflect the in vivo usage of reactions are generated. Probabilistic equations relate the determined intensities of the multiplet components to the relative abundance of intact carbon fragments (30) and thus allow derivation of intracellular carbon flux ratios (30–33). These data provide not only comprehensive insight into cellular metabolism but also inherent flux indications that can provide critical information for metabolic (not) flux analysis (27, 32).

The active pathways and the flux distribution in central carbon metabolism are critical components of a multidimensional

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TABLE 1. *E. coli* strains and plasmids used

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
MG1655	Wild-type K-12 strain (λ^- F^- *rph-1*)	1
JM101	[F^- *traD36 lacI*^{q} Δ(*lacZ*)*M15 proA*$^+$*B*$^+$ *supE thi* Δ(*lac-proAB*)]	43
PB25	Pyruvate kinase-deficient JM101 (*pykA::kan pykF::cat*)	25
ATCC 11303	Wild-type B strain; prototroph	American Type Culture Collection
KO20	Ethanol-producing ATCC 11303; chromosomal insertion of the *pet* operon (pyruvate decarboxylase and alcohol dehydrogenase II) of *Z. mobilis* into the pyruvate formate-lyase gene	23
Plasmids		
p*Trc99a*	*E. coli* expression vector	Pharmacia
pPPcc	p*Trc99a* derivative for expression of the artificial *E. coli pykF-pfkA* operon	6
pPYKbs	p*Trc99a* derivative for expression of the pyruvate kinase from *Bacillus stearothermophilus*	6

physiological representation of the organism, since this central backbone of metabolism provides energy, cofactor regeneration, and building blocks for biomass synthesis and controls the extent and nature of by-product excretion. A wide array of regulatory responses are embedded in this network on the transcriptional level as well as the protein level. The purpose of this complex regulatory structure is not yet fully elucidated, but the observed insensitivity of growth rates and extracellular fluxes to the overexpression of key enzymes suggests a homeostatic objective of the regulatory system (4, 8, 38).

In this study, we used METAFoR analysis to examine, at the level of flux ratios and operational pathways, how the central carbon physiology of *Escherichia coli* responds to genetic and environmental manipulations. In addition, we show the extent of variation in these facets of the central carbon network fluxome in several different standard laboratory strains. These METAFoR data show in detail how and under what conditions the *E. coli* central carbon metabolic network maintains flux ratio homeostasis and when significant alterations arise in both active pathways and flux ratios.

MATERIALS AND METHODS

Strains, plasmids, and media. Strains and plasmids used in this study are listed in Table 1. All batch cultivations were performed with a minimal medium containing 5 g of glucose per liter, 45 mM Na_2HPO_4, 22 mM KH_2PO_4, 10 mM NaCl, and 30 mM $(NH_4)_2SO_4$. The following components were sterilized separately and then added (per liter of final medium): 1 ml of 1 M $MgSO_4$, 1 ml of 0.1 mM $CaCl_2$, 1 ml of 1 mg of vitamin B_1 per liter (filter sterilized), and 10 ml of trace element solution containing (per liter) 0.55 g of $CaCl_2 \cdot 2H_2O$, 1 g of $FeCl_3$, 0.1 g of $MnCl_2 \cdot 4H_2O$, 0.17 g of $ZnCl_2$, 0.043 g of $CuCl_2 \cdot 2H_2O$, 0.06 g of $CoCl_2 \cdot 6H_2O$, and 0.06 g of $Na_2MoO_4 \cdot 2H_2O$. To ensure maintenance of plasmids, ampicillin was added to a final concentration of 25 mg/liter. The medium fed into the glucose-limited chemostat had the same composition as the batch medium, with the following exceptions (per liter): 3.6 g of glucose, 4.7 g of $Na_2HPO_4 \cdot H_2O$, 3.0 g of KH_2PO_4, 0.5 g of NaCl, and 1 g of NH_4Cl. To enforce nitrogen limitation, the concentration of glucose was increased to 4.5 g/liter, and the concentration of the sole nitrogen source, NH_4Cl, was reduced to 0.7 g/liter. Chemostat media were sterilized by passage through a 0.2-μm-pore-size filter, and 10-fold-diluted trace element solution was added after filtration to prevent losses via precipitation.

Batch and chemostat cultivations. All batch cultivations were performed at 37°C. Aerobic batch cultures were grown in 1-liter baffled shake flasks with 100 ml of medium on a gyratory shaker at 200 rpm. Anaerobic batch cultivations were performed with rubber-sealed glass flasks previously flushed with N_2 and incubated in a gyratory water bath (G76D; New Brunswick). Chemostats were operated at 37°C in a 1.3-liter bench-top fermentor (Hinengineering) with a working volume of 1.0 liter and a constant dilution rate (D) of 0.2 h^{-1}, meaning that the feed rate was 0.2 liter/h. The working volume was kept constant by removal of effluent from the center of the culture volume by use of a weight-controlled pump. The pH of the culture was maintained at 7.0 by automatic addition of 2.0 M NaOH with a pH controller and was verified periodically by off-line measurements. The airflow was maintained at 1 liter/min with filter-sterilized air by use of a volume flow meter, and the agitation speed was set to 1,200 rpm.

Labeling experiments with chemostats were initiated after the cultures appeared to reach a steady state, inferred from (i) at least five volume changes after adjustment to new conditions and (ii) stable optical density and oxygen and carbon dioxide concentrations in the fermentor effluent gas for at least two volume changes. The feed medium containing 3.6 (or 4.5 in the NH_4^+-limited experiment) g of unlabeled glucose per liter was then replaced by an identical medium containing 3.24 (4.05) g of glucose labeled by natural abundance per liter and 0.36 (0.45) g of [U-$^{13}C_6$]glucose (^{13}C, >98%; Isotech) per liter. Biomass samples for METAFoR analysis were taken after one volume change, so that 63% of the biomass was fractionally labeled according to the first-order washout kinetics that follow from assuming that the bioreactor contents are well mixed. Batch cultures were grown entirely in media supplemented with 4.5 g of glucose containing ^{13}C at natural abundance per liter and 0.5 g of [U-$^{13}C_6$]glucose per liter. Because the percentage of unlabeled biomass originating from the inoculum was well below 1% in the batch cultures, unlabeled biomass was subsequently neglected in the analysis of the ^{13}C-labeling patterns.

Analytical procedures. Cell growth during the cultivations was monitored by measuring the optical density at 600 nm (OD_{600}). For cellular dry weight (cdw) determination in selected cases, a known volume of fermentation broth was centrifuged for 10 min in prerweighed glass tubes at 4°C and 4,000 × *g*, washed once with water, and dried at 90°C for 24 h to a constant weight. Samples for extracellular metabolite analysis were centrifuged for 1 min at maximum speed in an Eppendorf tabletop centrifuge to remove the cells. Glucose and ethanol concentrations were determined enzymatically (Spectron CX5CE apparatus; Beckman) with kits supplied by the manufacturer. Acetate (and ethanol, in selected cases) was measured by gas chromatography (5890E chromatograph; Hewlett-Packard) with a Carbowax MD-10 column (Macherey-Nagel) and butyrate as an internal standard. Concentrations of oxygen and carbon dioxide in the feed medium and off gas of bioreactor fermentations were determined with a mass spectrometer (Prima 600; Fisons Instruments).

Determination of physiological parameters. In batch cultures, the exponential growth phase was identified by log-linear regression of biomass concentration versus time, with growth rate (μ) as the regression coefficient. The biomass yield on the substrate ($Y_{X/S}$) was determined as the coefficient of a linear regression of biomass concentration (X) versus substrate concentration (S) during the exponential growth phase. A predetermined correlation factor (OD_{600}, 0.33) was used to convert the OD_{600} values into cell concentrations for the calculation of specific conversion rates. The specific consumption rate for a substrate (q_S), e.g., glucose and O_2—defined as the differential change in S with time (t) normalized to the biomass concentration—was obtained as the coefficient of a linear regression of ΔS (the change in S) versus X divided by μ, on the basis of the relationship $S_{t_1} - S_{t_2} = \Delta S = X_{t_2}(q_S/\mu)$. The same relationship holds for the specific rate of formation of products (P), e.g., acetate and CO_2. This relationship is linear provided that μ and q_S are constant. In a steady-state chemostat, μ is constant and equals D. In batch cultures, maximum μ was constant during the exponential growth phase, and all specific rates from batch experiments reported here refer to the exponential phase.

In chemostat cultures, D and thus μ are constant; therefore, the consumption and production rates were determined from the difference between S or P in the feed medium (or air) and S or P in the effluent (or off gas). The relationship q_S or $q_P = \Delta S$ (or P) (D/X) normalized these rates to the steady-state concentration of biomass, generating the corresponding specific rates.

NMR sample preparation. For network topology and flux ratio analysis by NMR, a specified amount of culture was harvested and cells were centrifuged at 1,200 × *g* for 10 min at 4°C. The cell pellet was washed once with 20 mM Tris-HCl (pH 7.6) and centrifuged again. Washed pellets from chemostat cul-

tures were resuspended in the above buffer, and cells were disrupted by sonication on ice three times for 45 s each time at 20% output (XL-2020 sonication; Heat Systems). Cell debris was removed by centrifugation for 20 min at 9,000 × *g*. Sonication and centrifugation were repeated until cell lysis was virtually complete, as determined by visual inspection with a microscope. Small debris particles were removed by ultracentrifugation for 30 min at 33,000 × *g*. Cellular protein in the supernatant was precipitated overnight at −20°C after the addition of 60% (vol/vol) ethanol. The precipitate was resuspended in 6 ml of 6 M HCl and hydrolyzed by incubation in sealed Pyrex glass tubes for 24 h at 110°C. The hydrolysate was filtered through a 0.2-μm-pore-size filter and lyophilized. The dried material was dissolved in 600 μl of 20 mM deuterium chloride (DCl) in D_2O, incubated for 2 h at room temperature, centrifuged, and used for the NMR measurements. Washed pellets from batch cultures were directly resuspended in 6 M HCl and hydrolyzed.

NMR spectroscopy and data analysis. Proton-detected 2D ^{13}C-^1H heteronuclear single-quantum COSY was performed with the pulse sequence of Bodenhausen and Ruben (3), which ensures that ^1H-^{13}C scalar couplings do not affect the ^{13}C-^{13}C scalar coupling fine structure along the chemical shift $\omega_1(^{13}C)$ (24). Pulsed-field gradients were used for coherence pathway selection (± 40), and a 2-ms spin-lock pulse (24) was used to purge the magnetization arising from ^{12}C-bound protons and the residual ^2HOH signal. ^{13}C decoupling during data acquisition was achieved by use of the composite pulse decoupling scheme GARP (28), and quadrature detection in ω_1 was accomplished with States-TPPI (18). The spectra were recorded at a ^1H resonance frequency of 500 MHz by use of a Bruker DRX500 spectrometer; the sample temperature was 40°C. For each sample, two spectra were measured: one spectrum for the aliphatic resonances, with the ^{13}C carrier set to 42.5 ppm relative to 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt, and one spectrum for the aromatic resonances, with the ^{13}C carrier set to 125.9 ppm. The spectra of the aliphatic resonances were folded along $\omega_1(^{13}C)$ with a sweep width of 33.8 ppm. The measurement time was 4.5 h per spectrum (1,706 × 256 complex points; t_{1max}, 402 ms; t_{2max}, 102 ms; relaxation delay between scans, 2 s). The spectra of the aromatic resonances were recorded in about 2.5 h (720 × 512 complex points; t_{1max}, 392 ms; t_{2max}, 87 ms; relaxation delay between scans, 2 s). Before Fourier transformation with the program PROSA (12), the time domain data were multiplied in t_1 and t_2 with sine-bell windows shifted by π/2 (5). The digital resolutions after zero filling were 1.0 Hz/point along ω_1 and 2.4 Hz/point along the second frequency axis ω_2 for spectra of the aliphatic resonances and 0.6 Hz/point along ω_1 and 3.8 Hz/point along ω_2 for spectra of the aromatic resonances. One-dimensional ^1H NMR spectra (t_{1max} 1.022 s; 10-s relaxation delay between scans) were recorded to determine the overall degree of ^{13}C labeling in the amino acids from the satellites of isolated proton peaks, which corresponds to P_1 in the probabilistic equations of Szyperski (30).

The relative abundances of intact carbon fragments present in the eight principal intermediates that link central carbon metabolism to amino acid biosynthesis was determined from the intensities of the individual multiplet components in the ^{13}C-^{13}C scalar coupling fine structures (27, 30). Flux ratios through several key pathways in central metabolism were then calculated from the abundance of the fragments as described previously (31, 32).

Biochemical reaction master network of *E. coli*. As an initial step for METAFoR analysis, a biochemical master network that comprises all currently known reactions of central carbon and amino acid metabolism for the organism under investigation is constructed (30). For *E. coli*, this network (Fig. 1) was compiled from textbooks (11, 20) and Internet-accessible metabolic databases (16). Inspection of observed intact carbon fragments in the amino acids subsequently allows identification of active biosynthetic pathways (27, 30–32). Such analysis cannot distinguish between trioses that are generated via the methylglyoxal bypass or via the Entner-Doudoroff pathway, since these pathways generate fragment patterns that are indistinguishable from those emerging from the glycolysis and pentose phosphate (PP) pathways, respectively (30). Because the methylglyoxal bypass and the Entner-Doudoroff pathway were reported to be inactive for *E. coli* cells grown with glucose (11), they were not considered in the presently used network.

RESULTS

Analysis of glucose- and ammonia-limited chemostat cultures of wild-type *E. coli* MG1655. Continuous cultivation was performed with aerobic chemostats at a D (volumetric flow rate/working volume) of 0.2 h^{-1} under glucose- or ammonia-limited conditions, representing two largely different bioenergetic regimens. Carbon-sufficient (i.e., ammonia-limited) cultures are known to exhibit metabolic behavior that differs from that of carbon-limited cultures with respect to specific substrate consumption rate, maintenance requirements, and by-product secretion (21). This fact is reflected by the physiological data from the cultures described here (Table 2). When the ammonia-limited and the glucose-limited cultures were compared, marked increases were found in the specific glucose consumption rate (q_{glc}) and in the specific rates of production of acetate and pyruvate, while the specific oxygen consumption rate and CO_2 evolution rate varied little between the two different conditions; these results indicated that there are only minor changes in respiratory metabolism. The low steady-state biomass concentration in the ammonia-limited culture results from the low ammonia concentration used (13.1 mM). The residual glucose concentration in this culture was slightly above 1 g/liter.

For both cultures, the METAFoR data show evidence of two reactions that are generally considered to be inactive in *E. coli* grown in glucose-containing media (Fig. 2). These are the gluconeogenic conversion of oxaloacetate (OAA) to phosphoenolpyruvate (PEP) (Fig. 2G), catalyzed by the PEP carboxykinase (10, 11), and the conversion of malate (MAL) to pyruvate (PYR) (Fig. 2H and I) through the malic enzyme. Malic enzyme is normally required for growth on four-carbon compounds (11). Although the detected flux ratio of the latter reaction to all other reactions generating PYR is small, these data illustrate that the in vivo activity of reactions in central metabolism does not necessarily follow straightforward on-off paradigms (11, 20).

Cells harvested from the ammonia-limited culture clearly showed a fluxome pattern different from that of cells from the glucose-limited culture (Fig. 2). (i) Nearly double the fraction of OAA molecules was found to be derived from PEP (Fig. 2F), demonstrating an increased contribution from the anaplerotic PEP carboxylase reaction (9) and a corresponding decrease in MAL dehydrogenase activity. This anaplerotic reaction synthesizes the OAA that is required to replenish the pool of tricarboxylic acid (TCA) cycle intermediates, and its relative contribution therefore reflects the extent to which the TCA cycle is used for the biosynthesis of biomass components relative to energy generation (via oxidative phosphorylation) (11). (ii) A decrease in the fraction of PEP molecules originating from OAA was observed (Fig. 2G), providing evidence of reduced fluxes through the gluconeogenic PEP carboxykinase. (iii) An increase in the fraction of PYR derived from MAL was detected (Fig. 2H), indicating increased fluxes through the malic enzyme. (iv) A 50% reduction in the fraction of PEP molecules that were derived through at least one transketolase reaction was registered (Fig. 2B). This decrease would be consistent with an increased contribution from the glycolytic pathway relative to the PP pathway, suggesting that the higher catabolic fluxes in the ammonia-limited culture were mainly supported by glycolysis. (v) Less than 100% of the acetyl coenzyme A (ACoA) molecules were found to originate from PYR (Fig. 2J). This result can be explained by a dilution of the intracellular ACoA pool via exchange with the large, mostly unlabeled extracellular acetate pool that was detected in the ammonia-limited culture but not in the glucose-limited culture (Table 2). This result provides direct evidence for the presence of exchange fluxes between extracellular and intracellular acetate pools, as well as for the reversibility of the reactions connecting ACoA to acetate.

Analysis of wild-type batch cultures harvested at different growth phases. Chemostat experiments are the most suitable method of analyzing cells under steady-state conditions. On the other hand, a physiological steady state is also attained during the exponential growth phase in batch cultures, which are characterized by unrestricted growth at the maximum specific rate possible under the applied conditions. Because batch cultures enable more efficient parallel analysis of different strains, we adapted batch cultivations for METAFoR analysis of genetic effects on central carbon metabolism.

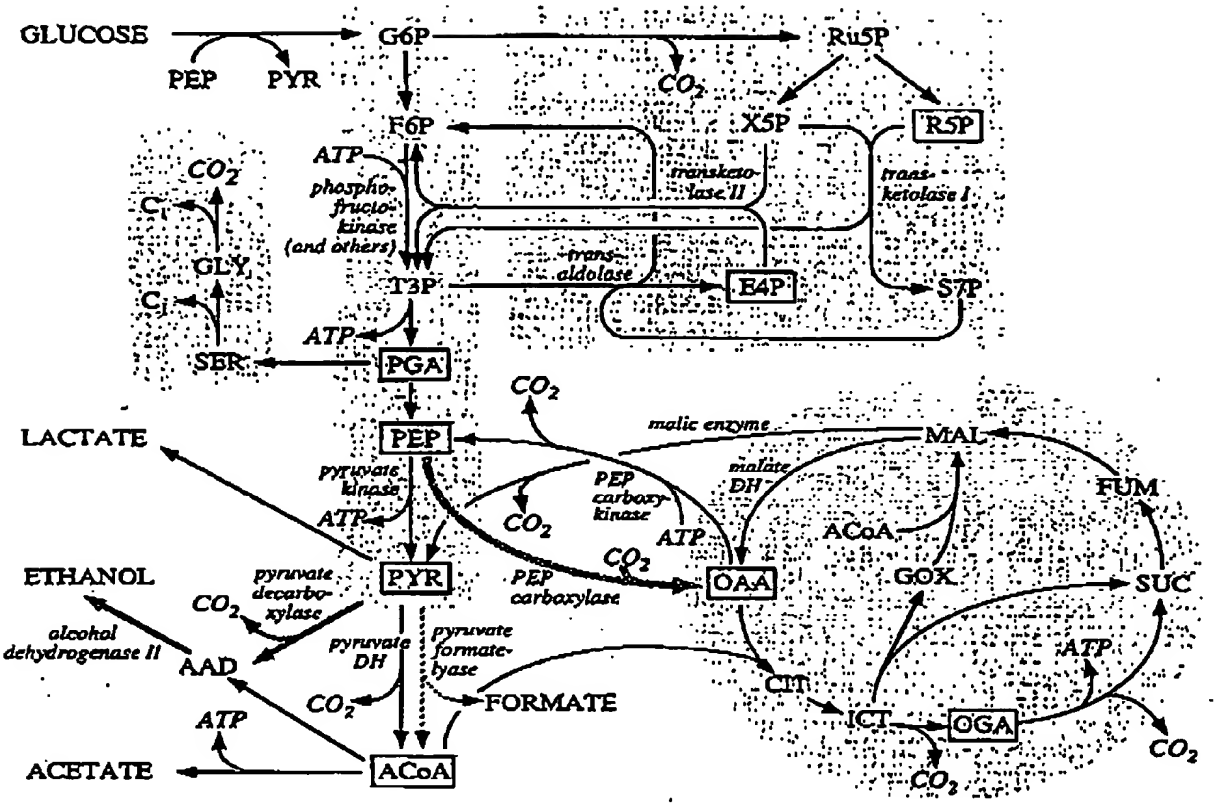

FIG. 1. Biochemical master network with reactions for identified genes or enzyme activities in *E. coli*. The information was compiled from the EcoCyc database (17) and other sources (11, 20). The reaction sets of glycolysis, the PP pathway, the TCA cycle (including the glyoxylate shunt), and C_1 metabolism are shaded, and enzymes catalyzing key reactions are indicated in italics. The hatched arrow highlights the TCA cycle-replumbing anaplerotic reaction, and the broken arrows indicate the anaerobic pyruvate formate-lyase, which is interrupted in strain KO20. The grey arrows indicate the reactions catalyzed by the enzymes encoded on the *pet* operon of *Z. mobilis*. The intact carbon fragment patterns of boxed metabolites were directly determined by $^{13}C/^1H$ COSY of proteinogenic amino acids. Abbreviations: F6P, fructose-6-phosphate; Ru5P, ribulose-5-phosphate; X5P, xylulose-5-phosphate; E4P, erythrose-4-phosphate; S7P, sedoheptulose-7-phosphate; PGA, 3-phosphoglycerate; SER, serine; GLY, glycine; AAD, acetaldehyde; CIT, citrate; ICT, isocitrate; OGA, oxoglutarate; SUC, succinate; GOX, glyoxylate; and DH, dehydrogenase.

The underlying principle of METAFoR analysis is the imprinting of central carbon network history into cell protein. This notion implies that, in a transient situation such as a batch cultivation, METAFoR results provide a time average over the interval of isotopic labeling of the biomass. To minimize changes in central carbon metabolism during the labeling period and to undertake the METAFoR analysis over a range of closely related physiological states, batch cultivations were initiated with mid-exponential-phase cultures at a low inoculum density (less than 1% the final culture volume) in medium containing 90% natural-abundance glucose and 10% [U-$^{13}C_6$]glucose. One set of experiments was undertaken to assess the sensitivity of METAFoR results to the sampling time of the labeled culture. These experiments also indicate

TABLE 2. Aerobic growth parameters of glucose- or ammonia-limited chemostat cultures of *E. coli* MG1655 at a D of 0.2 h^{-1}

Limitation	Biomass			Specific rate (mmol g^{-1} h^{-1}) of:				
	Concn (g [cdw] $liter^{-1}$)	Yield on:		Glucose consumption	Acetate formation	Pyruvate formation	O_2 consumption	CO_2 formation
		Glucose (g g of glucose $^{-1}$)	Nitrogen (g g of N^{-1})					
Glucose	1.45	0.40	5.5	2.8	0	0	8.3	8.7
Ammonia	0.55	0.17	3.0	6.4	4.4	0.8	10.6	9.6

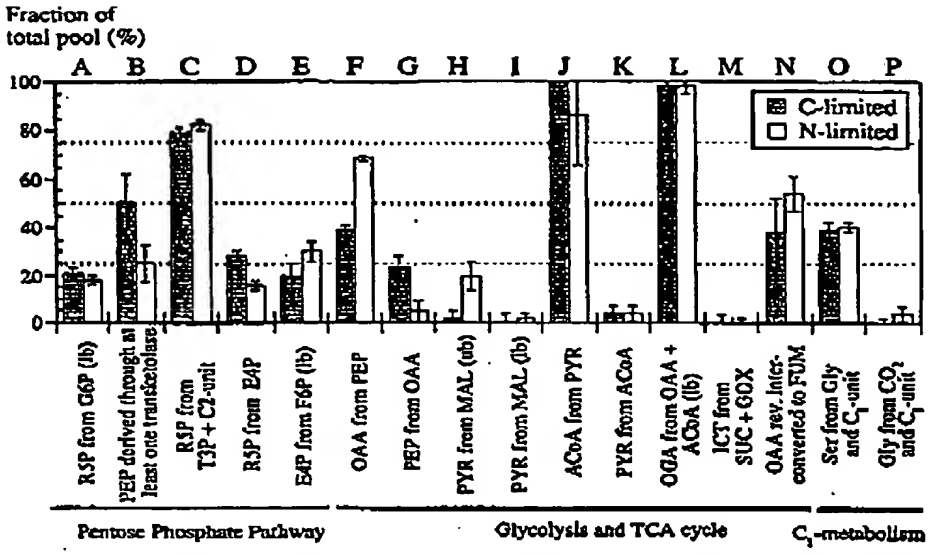

FIG. 2. Origins of metabolic intermediates (A to P) during aerobic growth of *E. coli* MG1655 in glucose-limited or ammonia-limited chemostats. In certain cases, the NMR data permit the determination only of upper bounds (ub) or lower bounds (lb) on the origin of intermediates. The experimental error (error bars) was estimated from the analysis of redundant ^{13}C scalar coupling fine structures and the signal-to-noise ratio of the ^{13}C-^1H COSY spectra by use of the Gaussian law of error propagation. The fraction of the total pool for a particular metabolite quantifies the ratio of this metabolite derived from a specified substrate to the sum of all other substrates that contribute to the pool of this metabolite. In cases where only two precursors contribute to the pool of a metabolite, the remaining fraction of the total pool can be attributed to the competing reaction. Abbreviations are explained in the text and in the legend to Fig. 1; rev., reversibly.

the extent to which significant changes in central metabolism occur between different sampling times.

Three batch cultivations of wild-type *E. coli* B strain ATCC 11303 were initiated in parallel and harvested in the mid-exponential phase, late exponential phase, and stationary phase (OD_{600}s 1, 2.6, and 4, respectively) (Fig. 3). METAFoR analysis indicates extremely similar flux ratio histories for all three samples, demonstrating that batch cultivations provide consistent central metabolic flux ratios (Table 3) when isotopic labeling of biomass is achieved throughout the exponential growth phase. Slight trends in some of the results, most notably in the fraction of OAA molecules derived from PEP, indicate adjustments in central metabolism that occur as the culture approaches and enters stationary phase. Overall, however, the present data indicate that time of harvest is not a critical parameter in the experiments used for this study. Therefore, in later experiments, all cultures were harvested at OD_{600}s between 0.9 and 1.2.

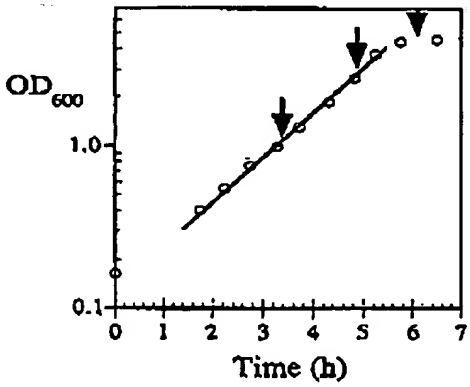

FIG. 3. Growth of *E. coli* ATCC 11303 in aerobic batch cultures. The line represents the best fit to the exponential growth phase data, and the arrows indicate the times of biomass sampling for the METAFoR analysis.

Analysis of aerobic batch cultures of wild-type and mutant *E. coli* strains during exponential growth. Two commonly used but genetically different *E. coli* strains, JM101 (a K-12 strain) and ATCC 11303 (a B strain), were grown in shake flask cultures under aerobic conditions for direct comparison of their carbon metabolism. Although ATCC 11303 grew somewhat faster (Table 4), the fluxome data for the two wild-type strains were almost identical (Fig. 4), suggesting that central metabolism is very similar in the two *E. coli* strains. There were, however, small changes in the origins of metabolites involved in the nonoxidative part of the PP pathway, from ribose-5-phosphate (R5P) to triose-3-phosphate (T3P) (Fig. 4B, C, and E), indicating that ATCC 11303 cells exhibit a higher degree of exchange through transketolase II.

In additional experiments, we studied the effects arising from knockout mutations which inactivate key central metabolism enzymes for each of the two wild-type strains. Pyruvate kinase-deficient PB25, which is otherwise isogenic with JM101, actually grew faster than its parent but exhibited similar biomass yield and specific glucose consumption rate (Table 4). While the overall METAFoR patterns were rather similar,

TABLE 3. Origin of intermediates in *E. coli* ATCC 11303 harvested during different growth phases from the cultures shown in Fig. 3[a]

Metabolite	Fraction of total pool (%) during the following growth phase:		
	Mid-exponential	Late exponential	Stationary
PP pathway			
R5P from G6P (lower bound)	29 ± 2	25 ± 2	24 ± 2
R5P from T3P + S7P	71 ± 3	75 ± 2	76 ± 2
Glycolysis and TCA cycle			
OAA from PEP	44 ± 1	40 ± 1	38 ± 1
PEP from OAA	7 ± 4	<7	6 ± 4
PYR from MAL (upper bound)	<5	<5	<5
PYR from MAL (lower bound)	<3	<3	<3
ACoA from PYR	>98	>98	>98
PYR from ACoA	0	0	0
OGA from OAA + ACoA	>98	>98	>98
ICT from SUC + GOX	<2	<2	0
OAA reversibly interconverted to FUM	46 ± 11	48 ± 10	50 ± 10
C_1 metabolism			
Ser from Gly + C_1 unit	7 ± 2	8 ± 2	9 ± 2
Gly from CO_2 + C_1 unit	4 ± 3	8 ± 3	9 ± 3

[a] The data were derived from a ^{13}C-^1H COSY spectrum centered on the aliphatic region; therefore, some data for the PP pathway that are accessible only through erythrose-4-phosphate incorporated in the aromatic rings of Trp or Phe could not be determined. See the text and the legend to Fig. 1 for abbreviations.

inactivation of both pyruvate kinase isoenzymes resulted in significant changes at the branch points between glycolysis and the TCA cycle (Fig. 4). Specifically, we found a higher fraction of OAA from PEP (Fig. 4F) and PYR from MAL (Fig. 4H and I) in the mutant. Apparently, the carbon flux from PEP to PYR is redistributed from pyruvate kinase to anaplerotic PEP carboxylase and the malic enzyme, allowing PB25 to generate sufficient pyruvate for fueling the TCA cycle via ACoA to generate energy (Fig. 1). These local changes provide evidence that considerable flexibility in *E. coli* central carbon metabolism permits the use of alternative pathways to compensate for knockout mutations.

Ethanol-producing *E. coli* KO20 is derived from ATCC 11303 and is characterized by a single chromosomal insertion of the artificial *pet* operon, which encodes the *Zymomonas mobilis* genes for alcohol dehydrogenase II and pyruvate decarboxylase, such that it disrupts the pyruvate formate-lyase gene (23). This strain grew faster and consumed glucose at a higher specific rate than both wild-type strains (Table 4). Although it primarily exhibited oxidative metabolism, it generated 50% more ethanol than did its parent. Despite these genetic and physiological differences, the METAFoR pattern of KO20 was, within experimental error, identical to that of ATCC 11303 (Fig. 4).

TABLE 4. Aerobic growth parameters of exponentially growing *E. coli* strains

Strain	μ (h^{-1})[b]	Y_{xs} ($g\ g^{-1}$)[c]	q_s ($g\ g^{-1}\ h^{-1}$)[c]	Concn (mM)[d] of	
				Acetate	Ethanol
PB25	0.64	0.30	2.2	2.0	0.4
JM101	0.60	0.30	2.0	1.5	0.4
ATCC 11303	0.65	0.29	2.2	1.6	1.0
KO20	0.70	0.28	2.5	1.6	1.5
KO20::pTrc99a	0.68	0.28	2.4	1.6	1.5
KO20::pPPcc	0.65	0.30	2.2	2.2	1.5
KO20::pPYKbs	0.74	0.29	2.3	1.5	2.0

[a] Standard deviation (SD), ±0.01.
[b] SD, ±0.02.
[c] SD, ±0.2.
[d] At an OD_{600} of 1.

Carbon metabolism during exponential growth in aerobic batch cultures of *E. coli* strains engineered for ethanol production. We chose ethanol-producing *E. coli* KO20 (23) as the host for a series of plasmids used for the expression of homologous and heterologous pyruvate kinases and phosphofructokinases (6). The expression of both genes was induced by use of isopropyl-β-D-thiogalactopyranoside (IPTG) (0.01 mM), leading to in vitro activities two- to ninefold above the control level, which was represented by the empty expression vector p*Trc99a* (Pharmacia). In terms of their growth physiology, all strains showed similar behaviors under aerobic conditions in batch cultures (Table 4). However, by-product formation was altered in the overexpression strains, with KO20::pPPcc generating more acetate and KO20::pPYKbs generating more ethanol than KO20. Again, the METAFoR patterns of all strains derived from ATCC 11303 were identical, within experimental error, to those of KO20 in Fig. 4 (data not shown). Thus, expression of the *pet* operon, medium-copy-number plasmid maintenance, and the overexpression of either a heterologous pyruvate kinase or the homologous phosphofructokinase do not appear to have a pronounced influence on the flux ratios accessible by current METAFoR analysis.

Analysis of anaerobic batch cultures of wild-type and mutant *E. coli* strains during exponential growth. The physiological differences among the wild-type and mutant *E. coli* strains under anaerobic conditions were larger than those observed in aerobiosis (Table 5). The two wild-type strains, JM101 and ATCC 11303, showed significant differences with respect to glucose consumption as well as acetate production rates, both of which were higher in ATCC 11303. Accordingly, the apparent biomass yield was lower in this strain. While pyruvate kinase-deficient PB25 grew slower than its parent, JM101, ethanol-producing KO20 grew fastest of all the strains investigated. Although the specific glucose consumption rates of JM101 and PB25 were identical, the latter was severely impaired in its ability to form the normal anaerobic end products ethanol and acetate. On the other hand, KO20 produced about fivefold more ethanol than any other strain and exhibited a higher glucose consumption rate and a lower biomass yield on glucose than its parent, ATCC 11303.

METAFoR analysis of the anaerobic cultures shown in Table 5 showed that OAA originated almost entirely from PEP via the anaplerotic reaction (Fig. 5F), illustrating the almost total absence of complete TCA cycle operation. This result concurs with the results of a recent study (7) and is consistent with earlier conclusions based on enzyme activity data, which indicated that, under anaerobic conditions, a branched, noncyclic TCA cycle pathway operates mainly to fulfill biosynthetic requirements (35). Because the fragments needed for tracing the activities of malic enzyme and PEP carboxykinase with METAFoR analysis do not appear under anaerobic conditions (27), these reactions are inaccessible to this analysis. Hence, although the experimental data conform to a bioreaction network devoid of malic enzyme (Fig. 5G), its activity cannot be excluded.

We cannot independently quantify the relative flux of OAA to PEP via PEP carboxykinase in anaerobically grown cells; therefore, METAFoR analysis cannot distinguish between OAA decarboxylation and PEP synthesis through the PP pathway. When ATCC 11303 (and its derivative, KO20) and JM101

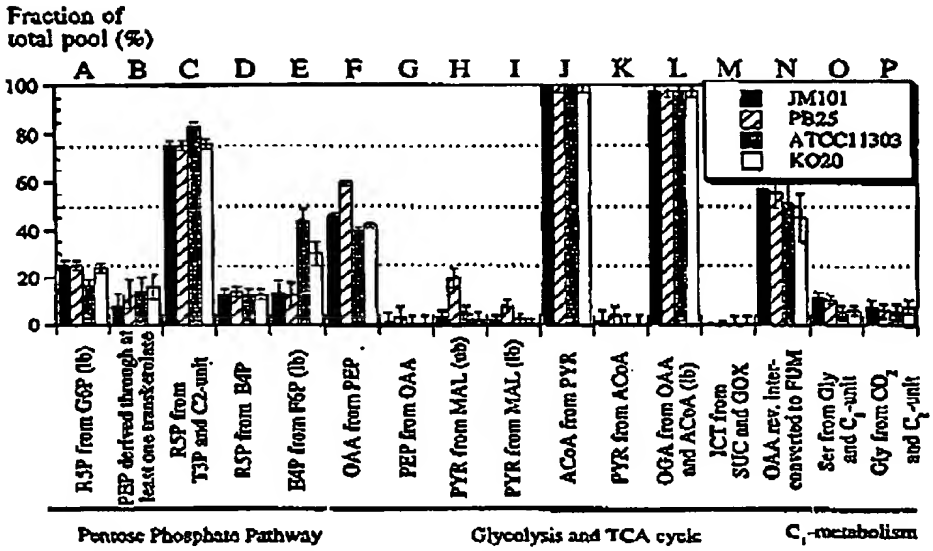

FIG. 4. Origin of metabolic intermediates (A to P) during aerobic exponential growth of various *E. coli* strains. For more details, see the legend to Fig. 2.

(and its derivative, PB25) are compared, the major difference in the fraction of PEP molecules that are not directly derived from glucose through glycolysis (Fig. 5B) may originate from OAA via decarboxylation, from a higher net flux through the PP pathway, or from increased exchange reactions in the PP pathway. However, the observation that the exchange fluxes in the PP pathway are very similar among all strains (Fig. 5C to E) indicates a major difference in flux through the PEP carboxykinase or PP pathway. The low but significant fraction of R5P found to originate from glucose-6-phosphate (G6P) (Fig. 5A) provides unambiguous evidence for the activity of the oxidative PP pathway during anaerobiosis.

In all cases, we observed a large fraction of PYR molecules that were interconverted at least once to ACoA (Fig. 5I), illustrating the in vivo reversibility of the anaerobic pyruvate formate-lyase reaction, as opposed to the irreversible aerobic pyruvate dehydrogenase reaction (11, 30) (see also Fig. 4K). However, in KO20 the pyruvate formate-lyase gene is disrupted and pyruvate conversion is achieved via the expression of a heterologous pyruvate decarboxylase. Because PYR exchange with ACoA in KO20 is almost identical to that in the reference strain, it appears either that the reversibility of this reaction is similar for pyruvate formate-lyase and pyruvate carboxylase or that an isoenzyme of pyruvate formate-lyase is responsible for this exchange. The uncharacterized product of the *E. coli yhaS* gene is highly homologous to pyruvate formate-lyase and thus may encode this activity.

Despite their considerably altered by-product formation characteristics, the two engineered strains, PB25 and KO20, exhibit central carbon metabolism surprisingly similar to that of their parent strains. This result can be seen in the fluxome data shown in Fig. 5, where the flux ratios are nearly identical in the parent strains and the modified strains, except for a reduced reversibility of the interconversion of OAA to fumarate (FUM) by the parent strains (Fig. 5L). This result provides further evidence for the homeostasis of carbon flux distribution in central metabolism following significant genetic modifications that impinge directly upon this metabolic subsystem.

DISCUSSION

This study provides novel insights into global metabolic network behavior. Variations resulting from different growth conditions and genetic backgrounds in *E. coli* are monitored by combined physiological and fluxome analyses. A particular strength of the METAFoR analysis used here is the ability to decipher the relative fluxes connecting the lower part of glycolysis with the TCA cycle, namely, the anaplerotic reaction and certain futile cycles which dissipate ATP. On the other hand, flux ratios of the oxidative versus the nonoxidative PP

TABLE 5. Anaerobic growth parameters of exponentially growing *E. coli* strains

Strain	μ (h^{-1})	$Y_{x/s}$ ($g\ g^{-1}$)	q_{glc} ($g\ g^{-1}\ h^{-1}$)	$q_{acetate}$[a] ($mM\ g^{-1}\ h^{-1}$)	q_{eth}[b] ($mM\ g^{-1}\ h^{-1}$)
PB25	0.23 ± 0.01	0.078 ± 0.003	3.1 ± 0.2	17 ± 1	7 ± 3
JM101	0.30 ± 0.01	0.097 ± 0.003	3.1 ± 0.2	27 ± 5	21 ± 3
ATCC 11303	0.33 ± 0.01	0.075 ± 0.003	4.4 ± 0.2	27 ± 5	54 ± 6
KO20	0.36 ± 0.01	0.066 ± 0.002	5.5 ± 0.2	118 ± 10	39 ± 6

[a] Specific ethanol formation rate.
[b] Specific acetate formation rate.

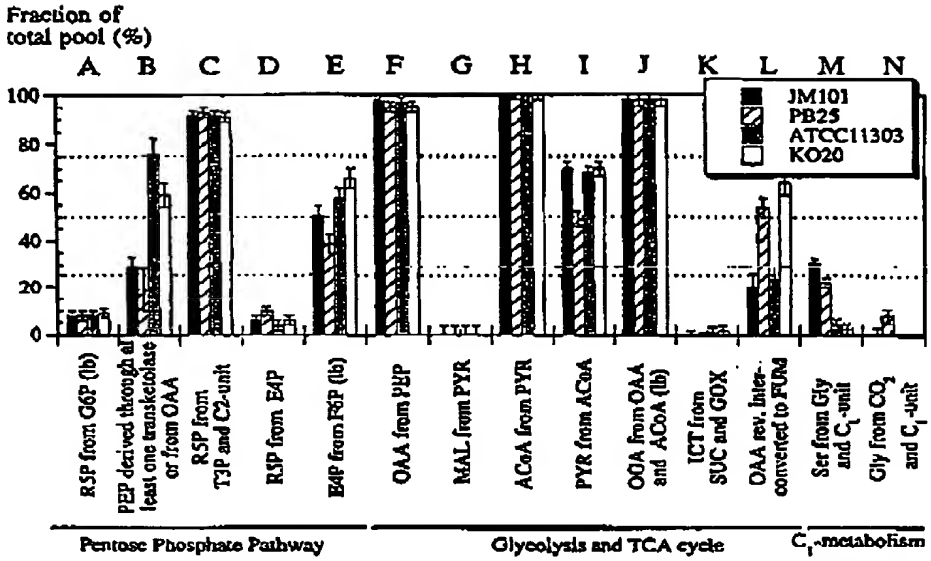

FIG. 5. Origin of metabolic intermediates (A to N) during anaerobic exponential growth of various *E. coli* strains. For more details, see the legend to Fig. 2.

pathways (R5P from G6P; Fig. 2A, 4A, and 5A) and glycolysis versus the PP pathway (PEP molecules derived through at least one transketolase reaction; Fig. 2B, 4B, and 5B) are accessible only as upper and lower bounds, because the pool of pentoses is rapidly and reversibly turned over by transaldolase and transketolase. The key findings are as follows. (i) Intracellular carbon flux ratios in the central metabolism of *E. coli* are affected only a little by enzyme overexpression and are flexible toward gene disruption. (ii) Of all central carbon fluxes, those in the TCA cycle change most significantly in response to changes in environmental conditions. (iii) Reactions mediated by the malic enzyme and PEP carboxykinase and previously considered to be absent during growth on glucose were identified. (iv) A novel regulation phenomenon in which futile cycling through at least one set of reactions is increased under conditions of a very low extracellular glucose concentration was evident.

Interstrain differences. The METAFoR pattern of exponentially growing aerobic *E. coli* revealed surprisingly few interstrain differences (Fig. 4), although major changes in physiological parameters are documented for the various strains used here. One would expect such physiological differences to be reflected in fluxes through central metabolism (Fig. 1), which provides energy, cofactor regeneration, and building blocks for biosynthesis. Strain KO20, for example, which expresses the *pet* operon of *Z. mobilis* alcohol dehydrogenase II and pyruvate decarboxylase and has pyruvate formate-lyase knockout mutations, was previously described to exhibit significantly altered by-product formation, with ethanol as the major product (23). Furthermore, overexpression of pyruvate kinase and phosphofructokinase, which are major control enzymes in the glycolytic pathway, were shown to have a profound effect on glucose catabolism in resting *E. coli* KO20. Specifically, an increased glucose consumption rate was found for KO20::pPYKbs harvested from aerobic precultures (13), and a large shift from ethanol to lactate formation was described for KO20::pPPcc harvested from anaerobic precultures (6).

Previously, pyruvate kinase deficiency was reported t alter growth kinetics under aerobic conditions (25). In the present study, JM101 and its pyruvate kinase-deficient derivative, PB25, showed few global changes in the METAFoR pattern. There was a small increase in the fraction of PEP molecules that were derived through at least one transketolase reaction (Fig. 4B), while all other exchange reactions in the PP pathway remained comparable (Fig. 4C to E). These results are consistent with the recent findings of Ponce et al. (26), who observed increased PP pathway activity in strain PB25 compared to strain JM101 by radiorespirometric analysis. Overall similarity in METAFoR patterns in response to genetic modifications seems to be a common feature of exponentially growing cells in aerobic cultures, consistent with the small differences in physiological parameters observed in this situation (Table 4). Under anaerobic conditions, where larger physiological changes were found, METAFoR analysis revealed more pronounced differences (Table 5 and Fig. 5; see also below).

Anaplerosis and the TCA cycle. METAFoR data provide information about the fraction of OAA molecules that originate from PEP (Fig. 2F, 4F, and 5F); this information quantifies the contribution of the TCA cycle-replenishing anaplerotic reaction to OAA generation, relative to that of MAL dehydrogenase in the TCA cycle (30–32). In the aerobic cultures studied here, the relative flux through anaplerotic PEP carboxylase is about 40%; the value for the ammonia-limited chemostat culture is increased to about 70% (Fig. 2F). Consistent with the extensive overflow metabolism seen in the ammonia-limited culture (Table 2), this increased anaplerosis indicates that, compared with the situation for the glucose-limited culture, a larger portion of the TCA cycle flux is used for biomass formation instead of energy generation. In the anaerobic regimen, the TCA cycle is reduced to a two-

branch pathway (30) and OAA is generated by anaplerosis only (Fig. 5F).

Futile cycling. In the glucose-limited chemostat, a significant fraction of PEP molecules were found to originate from OAA (Fig. 2G). This result indicates in vivo activity of gluconeogenic PEP carboxykinase, an enzyme that is required for growth on carbon sources that are metabolized via the TCA cycle and that has previously been considered to be inactive in cells grown on glucose (11). In principle, PEP molecules could also originate from OAA via the reverse reaction of anaplerotic PEP carboxylase, but based on thermodynamic considerations and the absence of $^{14}CO_2$ exchange with OAA in enzyme assays (37), this notion is highly unlikely. Hence, we have evidence of an ATP-dissipating futile cycle via PEP carboxylase and ATP-consuming PEP carboxykinase. At the same D under ammonia-limited conditions in the chemostat, this futile cycle appears to be significantly less active (Fig. 2G). Similar activity levels for this cycle were seen with *Bacillus subtilis* (27) grown in a chemostat under glucose limitation at a D of about 0.1 h^{-1}. In faster growing cells of *B. subtilis* in this chemostat, however, the contribution of this futile cycle was reduced, and the batch data presented here show it to be absent in exponentially growing *E. coli* (Fig. 4). On the other hand, for *Corynebacterium glutamicum*, a similar exchange between the PEP-PYR and OAA-MAL pools was described, not only for glucose-limited chemostat cultures (41) but also for batch cultures (29); however, it is not clear whether or not that pool exchange involved dissipation of ATP via a futile cycle. These data provide evidence for a metabolic regulation phenomenon in *E. coli* and *B. subtilis* in which futile cycle activity is less tightly controlled under extreme glucose limitation than under glucose excess, as in slow-growing chemostat cultures. It is tempting to speculate that this reduced control is caused by the extremely low extracellular glucose concentration and a concomitant reduction in catabolite repression. This hypothesis is also supported by the observation that PEP carboxykinase expression in *E. coli* is repressed by glucose (10).

In the anaerobically grown *E. coli* B strains ATCC 11303 and KO20, the high upper bound of the fraction of PEP originating from pentoses or OAA indicates a major difference in the metabolism of these strains and strains JM101 and PB25 (Fig. 5B). This difference could result from a higher flux either through the PP pathway or through PEP carboxykinase. From a physiological perspective, however, high fluxes through the oxidative PP pathway appear unreasonable, because anaerobic metabolism cannot reoxidize stoichiometrically formed NADPH with oxygen and reduced by-products were not detected. Therefore, it is more likely that a futile cycle involving PEP carboxykinase carries higher fluxes in ATCC 11303 and KO20. This scenario would be consistent with the observed higher specific rate of glucose catabolism and the reduced biomass yield compared to those in anaerobic *E. coli* JM101 cultures.

Exchange reactions. METAFoR analysis affords a qualitative assessment of several exchange fluxes (30). In the experiments analyzed here, the fraction of R5P molecules originating from T3P and a C_2 unit via the transketolase reaction was usually about 70 to 80% (Fig. 2C and 4C) and, under anaerobic conditions, even as high as 90% (Fig. 5C). In contrast, a much lower fraction of R5P molecules originated from erythrose-4-phosphate (Fig. 2D, 4D, and 5D), representing either an exchange via transaldolase or a recycling of PP pathway-generated fructose-6-phosphate to G6P and on to R5P. Similar insights into the PP pathway have previously been reported for batch cultures of *E. coli* K-12 and B strains by mass spectrometric analysis of ^{13}C-labeling patterns in the ribose moiety-containing nucleotides (15) and by METAFoR analysis (31). Rapid exchange of metabolite pools in the PP pathway was also described for *C. glutamicum* (19, 41). In *B. subtilis*, the exchange mediated by transketolase appears to be less significant, since about 50% of the R5P in slow-growing, glucose-limited chemostat cultures was found to contain intact C_5 fragments from the source glucose (27).

Three additional exchange fluxes can be assessed by the present methodology (30). First, the reversible interconversion of OAA to FUM was found to be invariant at about 50% in all cases, with the exception of JM101 and ATCC 11303 under anaerobic conditions (Fig. 2N, 4N, and 5L). Second, in C_1 metabolism the backward reaction from Gly and a C_1 unit to Ser was essentially negligible in aerobic batch cultures but was significant in anaerobic JM101 and PB25 batch cultures as well as in aerobic MG1655 continuous cultures (Fig. 4O and 5M). Third, in apparent contrast to earlier observations with amino acids obtained by hydrolysis of a purified recombinant protein (30, 42) we observed the reverse reaction in the glycine cleavage pathway (Gly from a C_1 unit and CO_2) under aerobic conditions but not under anaerobic conditions (Fig. 4P and 5N).

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